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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No. D-5639-C4
Anticipated Classification of this Application:
Class 436 Subclass 174
Prior Application: 08/556,951

BOX PATENT APPLICATION
ASSISTANT COMMISSIONER FOR PATENTS
Washington, D.C. 20231

Examiner: L. Alexander Art Unit: 1313

Sir: This is a request for filing a ☑ continuation ☐ divisional application under 37 CFR 1.53(b), of pending prior application **Serial No. 08/068,896** filed on **May 28, 1993**, of **Hutchens et al.** for **Methods and Apparatus for the Desorption and Ionization of Analytes**.

1. ☑ Enclosed is a complete copy of the prior application, including the oath or declaration as originally filed, and an affidavit or declaration verifying it as a true copy. (See 8 and 9, for drawing requirements.)

2. ☐ A verified statement to establish small entity status under 37 CFR 1.9 and 1.27:
   ☐ is enclosed
   ☐ was filed in the proper application and such status is still proper and desired (37 CFR 1.28(a)).

3. ☑ The filing fee is calculated below:

| CLAIMS AS FILED *IN THE PRIOR APPLICATION*, LESS ANY CLAIMS CANCELED BY AMENDMENT BELOW | | | | |
|---|---|---|---|---|
| | | Entity | | |
| ✓ | Fee for: | ☐ Small | ☑ Other | Amount |
| | Basic fee | $395.00 | $790.00 | 790.00 |
| | Each independent claim in excess of 3 | __ x $41.00 | 3 x $82.00 | 246.00 |
| | Each claim* in excess of 20 | __ x $11.00 | 11 x $22.00 | 242.00 |
| | Multiple dependent claim presented | __ x $135.00 | __ x $270.00 | 0.00 |
| *Including the total number of claims to which direct reference is made in all multiple dependent claims | | TOTAL FILING FEE ——> | | $1,278.00 |

4. ☑ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Account No. **06-2375 under Order No. 936611/D-5639-C4. A duplicate copy of this sheet is enclosed.**

5. ☐ A check in the amount of $ is enclosed.

6. ☐ Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

7. ☐ Amend the specification by inserting before the first line the sentence: -- This is a ☐ continuation, ☐ division.

8. ☐ Transfer the drawings from the prior application to this application and abandon said prior application as of the filing date accorded this application. **A duplicate copy of this sheet is enclosed for filing in the prior application file.** (May only be used if signed by person authorized by § 1.138 and before payment of base issue fee.)

9. ☑ New formal drawings are enclosed.

10. ☐ Priority of application Serial No. _____ filed on _____ in _____ is claimed under 35 U.S.C. 119.
☐ The certified copy has been filed in prior application Serial No. ___ , filed ___ .

11. ☑ The prior application is assigned of record to **Baylor College of Medicine**.

12. ☑ The power of attorney in the prior application is **Fulbright & Jaworski L.L.P., 1301 McKinney, Suite 5100, Houston, Texas 77010-3095.**

a. ☐ The power appears in the original papers in the prior application.
b. ☑ A new power of attorney is enclosed.
c. ☑ Address all future communications to **Fulbright & Jaworski L.L.P., 1301 McKinney, Suite 5100, Houston, Texas 77010-3095.**
(May only be completed by applicant, or attorney or agent of record.)

13. ☑ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

14. ☑ I hereby verify that the attached papers are a true copy of prior application Serial No. 08/068,896 as originally filed on March 28, 1993.

The undersigned declare further that all statements made herein of his or her own knowledge are true and that *all statements made on information and belief are believed to be true;* and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

7/28/98
Date

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☐ Filed under § 1.34(a)

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May 28, 1993

Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Re: 4-001; U.S. Patent Application
Invention: Method and Apparatus for Desorption and Ionization of Analytes
Inventors: T. William Hutchens and Tai-Tung Yip

Sir:

Transmitted herewith is the complete patent application of T. William Hutchens and Tai-Tung Yip for METHOD AND APPARATUS FOR DESORPTION AND IONIZATION OF ANALYTES, including the specification, claims, informal drawings, Declaration and Power of Attorney, Declaration Claiming Small Entity Status - Non-Profit Organization, return receipt postcard and check in payment of the filing fee.

The filing fee is calculated as follows:

| | |
|---|---|
| Basic Fee (Small Entity) | $355.00 |
| Independent Claims (in excess of 3) (3) | 111.00 |
| Dependent Claims (in excess of 20) (4) | 44.00 |
| Total Claims | 31 |
| Total Check enclosed | $510.00 |

The Commissioner is hereby authorized to charge any additional fees which may be required, or to credit any overpayment, to Deposit Account No. 22-0365.

Should the Commissioner wish to discuss this application with counsel for application, please telephone W. Ronald Robins at (713) 758-2452.

Sincerely,

W. Ronald Robins By Permission
W. Ronald Robins

0232:2350

Enclosures

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TRANSMITTED WITH CERTIFICATE OF EXPRESS MAILING

# METHOD AND APPARATUS FOR DESORPTION AND IONIZATION OF ANALYTES

The United States government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant No. 58-6250-1-003 awarded by the United States Department of Agriculture.

## BACKGROUND OF THE INVENTION

1. Field of the Invention.

This invention relates generally to methods and apparatus for desorption and ionization of analytes for the purpose of subsequent scientific analysis by such methods, for example, as mass spectrometry or biosensors. More specifically, this invention relates to the field of mass spectrometry, especially to the type of matrix-assisted laser desorption/ ionization, time-of-flight mass spectrometry used to analyze macromolecules, such as proteins or biomolecules.

2. Description of the Prior Art.

Generally, analysis by mass spectrometry involves the vaporization and ionization of a small sample of material, using a high energy source, such as a laser, including a laser beam. The material is vaporized from the surface of a probe tip by the laser beam, and in the process, some of the individual molecules are ionized by the gain of a proton. The positively charged ionized molecules are then accelerated through a short high voltage field and let fly into a high vacuum chamber, at the far end of which they strike a sensitive detector surface. Since the time of flight is a function of the mass of the ionized molecule,

the elapsed time between ionization and impact can be used to determine the molecule's mass which, in turn, can be used to identify the presence or absence of known molecules of specific mass.

All known prior art procedures which present proteins or other large biomolecules on a probe tip for laser desorption/ionization time-of-flight mass spectrometry rely on a crystalline solid mixture of the protein or other analyte molecule in a large excess of acidic matrix material deposited on the bare surface of a metallic probe tip. (The sample probe tip typically is metallic, either stainless steel, nickel plated material or platinum). Immobilizing the analyte in such a matrix was thought to be necessary in order to prevent the destruction of analyte molecules by the laser beam. The laser beam strikes the mixture on the probe tip and its energy is used to vaporize a small portion of the matrix material along with some of the embedded analyte molecules. Without the matrix, the analyte molecules are easily fragmented by the laser energy, so that the mass, and identity, of the original macromolecule is very difficult to determine.

This prior art procedure has several limitations which have prevented its adaptation to automated protein or other macrobiological molecules analysis. First, in a very crude sample it is necessary to partially fractionate (or otherwise purify the sample as much as possible) to eliminate the presence of excessive extraneous materials in the matrix/analyte crystalline mixture. The presence of large quantities of components may depress the signal of the targeted analyte. Such purification is time-consuming and expensive and would be very difficult to do in an automated analyzer.

Second, while the amount of analyte material needed for analysis by the prior art method is not large (typically in a picomole range), in some circumstances, such as tests on

pediatric patients, analyte fluids are available only in extremely small volumes (microliters) and may be needed for performing several different analyses. Therefore, even the small amount needed for preparation of the analyte/matrix crystalline mixture for a single analysis may be significant. Also, only a tiny fraction (a few thousandths or less) of analyte used in preparing the analyte/matrix mixture for use on the probe tip is actually consumed in the mass spectrometry analysis. Any improvement in the prior art procedure which made it possible to use much less analyte to conduct the test would be highly advantageous in many clinical areas.

Third, the analyte protein, or other macromolecule, used in preparing the analyte matrix for use on the probe tip is not suitable for any subsequent chemical tests or procedures because it is bound up in the matrix material. Also, all of the matrix material used to date is strongly acidic, so that it would affect many chemical reactions which might be attempted on the mixture in order to modify the analyte molecules for subsequent examination. Any improvement in the procedure which made it possible to conduct subsequent chemical modifications or reactions on the analyte molecules, without removing them from the matrix or the probe tip, would be of enormous benefit to researchers and clinicians.

Additional limitations in the prior art included problems with matrix use such as:

(1) formation of analyte-matrix complex (referred to as "matrix adduct" which interferes with the accuracy of analyte measurement;

(2) inability to wash away contaminants present in analyte or matrix (e.g., other proteins or salts);

(3) formation of analyte-salt ion adducts;

(4) less than optimum solubility of analyte in matrix;

(5) signal (molecular ion) suppression "poisoning" due to simultaneous presence of multiple components; and

(6) selective analyte desorption/ionization.

There are a number of problems and limitations with the prior art methods. Prior investigators, including Karas and Hillenkamp have reported a variety of techniques for analyte detection using mass spectroscopy, but these techniques suffered because of inherent limitations in sensitivity and selectivity of the techniques, specifically including limitations in detection of analytes in low volume, undifferentiated samples. The "Hillenkamp-Karas" articles that pertain to this field of invention are:

1. Hillenkamp, "Laser Desorption Mass Spectrometry: Mechanisms, Techniques and Applications"; *Bordeaux Mass Spectrometry Conference Report*, 1988, pages 354-362.

2. Karas and Hillenkamp, "Ultraviolet Laser Desorption of Proteins Up to 120,000 Daltons", *Bordeaux Mass Spectrometry Conference Report*, 1988, pages 416, 417.

3. Karas and Hillenkamp, "Laser Desorption Ionization of Proteins With Molecular Masses Exceeding 10,000 Daltons", *Analytical Chemistry*, 60. 2299, July 1988.

4. Karas, Ingendoh, Bahr and Hillenkamp, "UV-Laser Desorption/Ionization Mass Spectrometry of Femtomol Amounts of Large Proteins", *Biomed. Environ. Mass Spectrum* (in press).

The use of laser beams in time-of-flight mass spectrometers is shown, for example, in U.S. Pat. Nos. 4,694,167; 4,686,366, 4,295,046, and 5,045,694, incorporated by reference.

The first successful molecular mass measurements of *intact* peptides and small proteins (only up to about 15 kDa) by any form of mass spectrometry were made by

bombarding surfaces with high energy particles (plasma desorption and fast atom bombardment mass spectrometry); this breakthrough came in 1981 and 1982. Improvements came in 1985 and 1986, however, yield (signal intensities), sensitivity, precision, and mass accuracy remained relatively low. Higher molecular mass proteins (about 20 to 25 kDa) were not observed except on rare occasions; proteins representing average molecular weights (approximately 70 kDa) were not ever observed with these methods. Thus, evaluation of most proteins by mass spectrometry remains unrealized.

In 1988, Hillenkamp and his coworkers used UV laser desorption time-of-flight mass spectrometry and discovered that when proteins of relatively high molecular mass were deposited on the probe tip in the presence of a very large molar excess of an acidic, UV absorbing chemical matrix (nicotinic acid) they could be desorbed in the intact state. This new technique is called *matrix-assisted* laser desorption/ionization (MALDI) time-of-flight mass spectrometry. Note that laser desorption time-of-flight mass spectrometry (without the chemical matrix) had been around for some time, however, there was little or no success determining the molecular weights of large intact biopolymers such as proteins and nucleic acids because they were fragmented (destroyed) upon desorption. Thus, prior to the introduction of a chemical matrix, laser desorption mass spectrometry was essentially useless for the detection of specific changes in the mass of intact macromolecules (see below). Note that the random formation of matrix crystals and the random inclusion of analyte molecules in the solid solution is prior art.

## SUMMARY OF THE INVENTION

The primary object of the invention is to provide improved methods, materials composition and apparatus for coupled adsorption, desorption and ionization of multiple or selected analytes into the gas (vapor) phase, preferably for use in conjunction with mass spectrometry of biomolecules and other macromolecules, as well as by means of analytic detection other than mass spectrometry. The invention incudes a flexible variety of options for presenting surfaces with attached energy absorbing molecules, defined reaction sites, and affinity reagents for the capture,e transfer, and/or the desorption of analytes before and after a sseries of chemical, physical, and/or enzymatic modifications performed *in situ*.

Another object is to provide such a method and apparatus for affinity-directed detection of analytes, including desorption and ionization of analytes in which the analyte is not dispersed in a matrix solution or crystalline structure but is presented within, on or above an attached surface of energy absorbing "matrix" material through molecular recognition events, in a position where it is accessible and amenable to a wide variety of chemical, physical and biological modification or recognition reactions.

The probe surface with and without bonded energy absorbing molecules, referred to as the sample presenting surface can be composed of a variety of materials, including porous or nonporous materials, with the porous materials providing sponge-like, polymeric, high surface areas for optimized adsorption and presentation of analyte.

These surface materials can be substituted (at varying densities) with chemically bonded (covalent or noncovalent) affinity adsorption reagents and/or chemically bonded (i.e., immobilized) energy absorbing molecules (bound "matrix" molecules). The geometry of the sample presenting surface can be varied (i.e., size, texture, flexibility, thickness, etc.)

to suit the need (e.g., insertion into a living organism through spaces of predetermined sizes) of the experiment (assay).

Another object is to provide such a method and apparatus in which the analyte material is chemically bound or physically adhered to a substrate forming a probe tip or other sample presenting surface.

A further object is to provide means for the modification of probe or sample presenting surfaces with energy-absorbing molecules to enable the successful desorption of analyte molecules without the addition of exogenous matrix molecules as in prior art.

A further object is to provide the appropriate density of energy-absorbing molecules bonded (covalently or noncovalently) in a variety of geometries such that mono layers and multiple layers of attached energy-absorbing molecules can be used to facilitate the desorption of analyte molecules of varying masses. The optimum ratio of adsorbed or bonded energy-absorbing molecules to analyte varies with the mass of the analyte to be detected. A further object is to modify the sample presenting surface with such energy-absorbing molecules where the composition of the probe or sample presenting surface is other than the metal or metallic surfaces as described in prior art. Separate from the chemical and/or physical modification of the probe surface with energy absorbing molecules is the modification of these surfaces with affinity reagents, both chemical and/or biological, for the specific purpose of capturing (adsorbing) specific analyte molecules or classes of analyte molecules for the subsequent preparation, modification, and successful desorption of said analyte molecules.

A further object is to provide all combinations of surfaces modified with energy-absorbing molecules and/or affinity-directed analyte capture devices to enable the selective

and/or nonselective adsorption of analytes and the subsequent desorption either with or without requiring the subsequent addition of additional matrix molecules. It is important to note that the surfaces modified with affinity reagents for the capture of analytes are more useful than the underivitized sample surfaces described in prior art even when the deposition of energy-absorbing molecules (that is matrix) is as described in prior art. Because of the advantages in the ability to remove contaminating substances from the adsorbed analyte molecules and because of the ability to modify adsorbed analyte molecules without (or before) added matrix.

A further object is to provide such a method and apparatus in which the substrate forming the probe tip or other sample presenting surface is derivatized with one or more affinity reagents (a variety of densities and degrees of amplification) for selective bonding with predetermined analytes or classes of analytes.

A further object is to provide methods and apparatus for using probe tips having surfaces derivatized with affinity reagents and containing laser desorption matrix material (chemically bonded to surface or not) which may be used to isolate target analyte materials from undifferentiated biological samples such as blood, tears, urine, saliva, gastrointestinal fluids, spinal fluid, amniotic fluid, bone marrow, bacteria, viruses, cells in culture, biopsy tissue, plant tissue or fluids, insect tissue or fluids, etc.

Because of the new and preferred method for presentation and desorption of selected analytes, a further object is to use analyte detection methods other than the generic electron multipliers typically used in mass spectrometric devices. This would include but would not be limited to detection films/plates for the qualitative or quantitative evaluation of fluorescent or radio-labeled analytes or analyte molecule complexes.

A further object is to provide such a system in which the affinity reagent chemically bonds or biologically adheres to the target analyte or class of analytes.

A further object is to use existing and new solid phase affinity reagents (e.g., small diameter porous or nonporous beads of crosslineated polymer with attached molecular capture devices) designed for the (1) capture (adsorption of one or more analytes, (2) the preparation of these captured analytes (e.g., washing with $H_2O$ or other buffered or nonbuffered solutions to remove contaminants such as salts, multiple cycles of washing, such as with polar organic solvent, detergent-dissolving solvent, dilute acid, dilute base or urea), and (3) most importantly, the direct transfer of these captured and prepared analytes to the probe surface for subsequent analyte desorption (for detection, quantification and/or mass analysis).

A further object is to provide such a system in which the predetermined analytes are individual biomolecules or other macromolecules or combinations of adjoined molecules (i.e., complexes).

A still further object is to provide such a method and apparatus in which the matrix materials used are not strongly acidic, as in prior art matrices, but are chemically modified into the slightly acidic, neutral pH or strongly basic range of pH.

A further object is to provide such a system in which the matrix material has a pH above 6.0.

A still further object is to provide a method and apparatus for desorption and ionization of analytes in which unused portion of the analytes contained on the presenting surface remain chemically accessible, so that a series of chemical and/or enzymatic or other treatments (e.g., discovery of analyte-associated molecules by molecular recognition) of the

analyte may be conducted on the probe tip or other presenting surface, *in situ*, followed by sequential analyses of the modified analyte by mass spectrometry. In one case (i.e., repetitive sequential analyses) the analyte is adsorbed to the sample presenting surface and can be treated (modified *in situ* after the excess free matrix is removed (i.e., washed away). Matrix can be added back before analysis by mass spectrometry. Using this procedure, an analyte can be repeatedly tested for a variety of components by removing one matrix, modifying the analyte sample, re-applying the same or different matrix, analyzing the sample, etc.

A further object is to provide a method and apparatus for the combined chemical and/or enzymatic modifications of target analytes for the purpose of elucidating primary, secondary, tertiary, or quaternary structure of the analyte and its components.

A still further object is to provide such a method and apparatus in which the probe tips or other sample presenting surfaces are formed of a variety of materials, including electrically insulating materials (porous and nonporous), flexible or nonrigid materials, optically transparent materials (e.g., glass, including glass of varying densities, thicknesses, colors and with varying refractive indices), as well as less reactive, more biocompatible materials (e.g., biopolymers such as agarose, dextran, cellulose, starches, peptides, and fragments of proteins and of nucleic acids such as DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). These surfaces can be chemically modified by the attachment of energy-absorbing molecules and/or affinity directed analyte capture molecules.

Another object is to provide a method and apparatus for desorption and ionization of analyte materials in which cations other than protons ($H^+$) are utilized for ionization of analyte macromolecules.

Note that the laser or light source used to convey energy to the probe surface can employ a wavelength(s) that is(are) not fixed but can be varied according to the wavelength absorbed by the matrix (whether the matrix is added in the free form or is chemically bonded to the probe (sample presenting) surface). For this procedure, a variety of wavelengths (10 or more) defined by absorbance of matrix or energy absorbing surface can be utilized.

Another object is to provide such a method and apparatus in which the probe tips or other sample presenting surfaces used for laser desorption/ionization time-of-flight mass spectrometry are magnetized and in which the matrix, affinity directed absorption molecules and/or analyte materials are magnetically adhered to such magnetized surface.

A further object is a method and apparatus in which the matrix and/or analyte materials are adhered by any variety of chemical mechanisms to the sample presenting surface.

A further objective is to provide energy-absorbing molecules which have been incorporated into other chemical structures (e.g., chemical or biological polymers) for the deposition (covalent or noncovalent) onto the sample presenting surface in a way that enables repetitious analyte desorption events without interference with chemical and/or enzymatic modifications of the analyte molecule(s) performed *in situ*.

Another object is to provide sample presenting surfaces in a variety of sizes and configurations (up to 4"x4") with multiple (up to 10,000 or more) spots (including spots down to <0.001 inch diameter) of affinity reagents arranged in predetermined arrays for the selective adsorption of numerous different analytes (e.g., clinical chemical marker proteins)

to enable a wide spectrum sampling of the macromolecular composition of biological samples/fluids.

As shown more fully below, the present invention overcomes limitations and disabilities of the prior art by providing probe tips or sample plates whose surfaces have been derivatized with biospecific affinity reagents which will selectively bind specific groups or types of biomolecules or other analytes out of an undifferentiated sample (such as blood or urine). Appropriate selection of the affinity reagents used to derivatize the probe tip surface therefore makes possible the selection from the undifferentiated sample and binding to the probe tip of the specific types or groups of biological or other macromolecules under investigation, or subsequent examination (e.g., quantification and/or structure elucidation) by mass spectrometry. This has the advantage of achieving both the purification of the analyte sample previously required and the effect of concentrating the analyte. It reduces by a factor of 1,000 to 100,000 the amount of analyte needed for the mass spectrometry examination, since only the macromolecules which attach to the biospecific affinity reagents are removed from the analyte sample, and these can be sequestered on predetermined areas of the probe tips or sample plates that are even less than the laser spot size.

It also has been found that the probe tips used in the process of the invention need not be metal or metal-coated, as with prior art procedures. Research involved in the invention has involved glass and synthetic polymer surfaces such as polystyrene, polypropylene, polyethylene, polycarbonate and other polymers including biopolymers, for the probe tips which have been covalently or noncovalently derivatized for immobilization of specific reagents that will direct the selective adsorption of specific analytes. These surfaces will include immobilized metal ions, immobilized proteins, peptides, enzymes, and

inhibitor molecules, immobilized DNA and RNA, immobilized antibodies, immobilized reducing agents, immobilized carbohydrates and lectins, immobilized dyes and immobilized protein surface domains involved in molecular recognition (e.g., dimerization domains and subunits). Some of the chemical and surface structures are as yet unknown.

The preferred probe tip, or sample plate, for selective adsorption/presentation of sample for mass analysis are (1) stainless steel (or other metal) with a synthetic polymer coating (e.g., cross-linked dextran or agarose, nylon, polyethylene, polystyrene) suitable for covalent attachment of specific biomolecules or other nonbiological affinity reagents, (2) glass or ceramic, and/or (3) plastic (synthetic polymer). The chemical structures involved in the selective immobilization of affinity reagents to these probe surfaces will encompass the known variety of oxygen-dependent, carbon-dependent, sulfur-dependent, and/or nitrogen-dependent means of covalent or noncovalent immobilization. The methods and chemical reactions used in producing such surfaces derivatized with biospecific affinity reagents already are known by those skilled in the art. Two features of the invention, however, are (1) the specific size and localization of the derivatized surface with respect to the laser beam and (2) the affinity directed presentation of specific analyte molecules (e.g., macromolecule or biopolymer) at a defined surface density or local concentration required for the efficient detection by laser desorption/ionization time-of-flight mass spectrometry. This can be accomplished by arranging the affinity adsorption "spots" (0.005 to 0.080 inch diameter) on the probe surface in a defined manner (400 to 1,000 spots could be placed on a surface about the size of a glass slide).

An additional discovery involves the fact that pH modified chemical matrices can be used on these surfaces to facilitate desorption/ionization without disruption of conditions

necessary for subsequent sample modification. As discussed above, prior art matrix materials used for biomolecular mass spectrometry are acidic. The exact chemical structure of the pH-modified matrices still are unknown. However, by suitable neutralization of the matrix material, it can be made largely passive to subsequent chemical or enzymatic reactions carried out on the analyte molecules presented on the derivatized probe tip surface by the biospecific affinity reagents. This makes possible the carrying out of chemical reactions on the analyte molecules presented on the probe tips. Since only a small fraction of the analyte molecules are used in each desorption/mass spectrometer measurement, a number of sequential chemical and/or enzymatic modifications of the samples, *in situ*, on the probe tips, and subsequent analysis of the modified samples by mass spectrometry, can be carried out on the same probe tips in order to more accurately determine exactly what molecule is present, or other characteristics or information about the molecule, including its structure.

Finally, even when these matrix molecules are immobilized on a probe tip surface, the analyte deposited on such a surface can be desorbed with a laser beam. This circumvents the contamination of the analyte by the matrix molecules. As a particular feature of the invention, we have shown that some energy absorbing molecules that do not work as "matrix" molecules when added to analytes as a solution of free molecules (as in prior art) do indeed work well to facilitate the desorption of intact analyte molecules after being immobilized.

It seems likely that these improvements in the procedure will enable bioanalytical and medical instrument manufacturers to develop a machine for the automated evaluation of

a single protein sample deposited on a surface and modified with numerous chemical and/or enzymatic reactions performed *in situ*.

## BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects and advantages of the invention will be apparent from the following specification and from the accompanying drawings.

Figure 1A is a mass spectrum of peptide mixtures using sinapinic acid pH 6.5 as the matrix. Figure 1B is a mass spectrum of the same peptide mixtures after *in situ* addition of $CuSO_4$.

Figure 2A is a mass spectrum of human casein phosphopeptide (R1-K18 + 5P) using sinapinic acid pH 6.5 as the matrix. Figure 2B shows the mass spectrum of the same peptide after *in situ* alkaline phosphatase digestion for 5 and 10 min respectively. Figure 2D shows the mass spectrum of the same peptide using dihydroxybenzoic acid pH 2 as the matrix, after *in situ* alkaline phosphatase digestion for 10 min.

Figure 3 is a mass spectrum histidine-rich glycoprotein $(GHHPH)_5G$ peptide using sinapinic acid pH 6.2 as the matrix before and after *in situ* digestion with carboxypeptidase P.

Figure 4 is a composite mass spectra of peptide mixtures using sinapinic acid as the matrix on glass, polypropylene-coated steel, polystyrene-coated steel and nylon probe tips.

Figure 5A is a mass spectrum of peptides unadsorbed by TSK SW-IDA-Cu(II). Figure 5B is a mass spectrum of peptide adsorbed by TSK SW-IDA-Cu(II). Figure 5C is a mass spectrum of the same peptide adsorbed on TSK SW-IDA-Cu(II) after water wash.

Figure 6 is a mass spectrum of myoglobin ($\leq$8 fmole) affinity-adsorbed on TSK SW-IDA-Cu(II).

Figure 7A is a mass spectrum of proteins/peptides in infant formula. Figure 7B is a mass spectrum of phosphopeptides in the same sample affinity-adsorbed on Sepharose-TED-Fe(III). Figure 7C is a mass spectrum of proteins/peptides in gastric aspirate of preterm infant. Figure 7D is a mass spectrum of the phosphopeptides in the same sample adsorbed on Sepharose-TED-Fe(III).

Figure 8 (bottom) is a mass spectrum of rabbit anti-human lactoferrin immunoglobin affinity adsorbed on paramagnetic Dynabead-sheep anti-rabbit IgG. Figure 8 (top) is a mass spectrum of human lactoferrin and rabbit anti-human lactoferrin IgG complex affinity adsorbed on paramagmetic Dynabead-sheep anti-rabbit IgG.

Figure 9 is a mass spectrum of human lactoferrin affinity adsorbed on a single bead of agarose-single-stranded DNA deposited on a 0.5 mm diameter steel probe tip.

Figure 10 is the mass spectrum of human lactoferrin affinity adsorbed from urine on agarose-single-stranded DNA.

Figure 11A is a mass spectrum of human gastrointestinal fluid. Figure 11B is a mass spectrum of trypsin in the same sample affinity adsorbed on AffiGel 10-soybean trypsin inhibitor.

Figure 12A is a mass spectrum of human serum proteins. Figure 12B is a mass spectrum of human serum albumin in the same sample affinity adsorbed on agarose-Cibacron blue.

Figure 13 is a drawing of the surface bound cinnamamide; R represents the surface plus cross-linear.

Figure 14A is a mass spectrum of peptide mixtures on surface bound cinnamamide. Figure 14B shows the mass spectrum of the same peptide mixtures on free cinnamamide.

Figure 15 is a drawing of the surface bound cinnamyl bromide; two structural forms are possible; R represents the surface plus cross-linear.

Figure 16A is a mass spectrum of peptide mixtures on surface bound cinnamyl bromide. Figure 16B is a mass spectrum of the same peptide mixtures on free cinnamyl bromide.

Figure 17 is a drawing of the surface bound MAP-dihydroxybenzoic acid; R represents the surface plus cross-linear.

Figure 18 is a mass spectrum of peptide mixtures on surface bound MAP alone (control surface). Figure 18B is a mass spectrum of the same peptide mixtures on surface bound MAP-dihydroxybenzoic acid.

Figure 19A is a mass spectrum of myoglobin on surface bound cyanohydroxycinnamic acid. Figure 19B is the same mass spectrum in the low mass region.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

### DETAILED DESCRIPTION

Usage of conventional matrix in aqueous, pH-neutralized form

Examples:

Sinapinic acid (dimethoxy hydroxycinnamic acid) (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin) 20 mg/ml water suspension (intrinsic pH 3.88)

Dihydoxybenzoic acid (Aldrich) 20 mg/ml water (intrinsic pH 2.07)

Cyanohydroxycinnamic acid (Aldrich) 20 mg/ml water suspension (intrinsic pH 3.3)

each titrated with triethylamine (Pierce, Rockford, IL) to pH 6.5, 7.2 and 6.5 respectively

2 ul of the matrix solution was mixed with 1 ul of sample and allowed to air dry

1. A mixture of synthetic peptides - human histidine-rich glycoprotein peptide $(GHHPH)_2G$, $(GHHPH)_5G$, human estrogen receptor dimerization domain (D473-L525) with neutralized sinapinic acid as the matrix, in the absence and presence of Cu(II). FIGURE 1 showed the *in situ* metal-binding properties of the peptides under neutralized condition.

2. Casein phosphopeptide (R1-K18 + 5P) with sinapinic acid pH 6.5. Followed by *in situ* alkaline phosphatase (0.5 ul, Sigma) digestion for 10 min at room temperature. Similar *in situ* digestion on the same peptide with dihydoxybenzoic acid (prepared in 30% methanol/0.1% trifluoroacetic acid) was used as control. FIGURE 2 showed the more efficient enzymatic dephosphorylation under neutralized condition.

3. Mixture of synthetic peptides as in 1 with sinapinic acid pH 6.2, followed by *in situ* carboxypeptidase P (1 ul, Boehringer Mannheim Corp, Indianapolis, IN, 20 ug/50 ul) digestion for 30 min at room temperature. FIGURE 3 showed preferential removal of C-terminal amino acid from histidine-rich glycoprotein peptide. Also showed unambiguous C-terminal determination even in peptide mixtures.

Usage of probe tip (surface) materials (composition) other than stainless steel or platinum for sample deposition

Examples:

Molten polypropylene or polystyrene was deposited on stainless steel probe tip so as to cover it completely

Solid glass rod (1.5 mm dia) was cut into 1 cm segments and inserted into stainless steel probe tip support

Solid nylon (Trimmer line, 1.5 mm dia, Arnold, Shelby, Ohio) was cut into 1 cm segments and inserted into stainless steel probe tip support

Magnetic stir bars (1.5 x 8 mm, teflon coated, Curtin Matheson Scientific, Inc., Houston TX) inserted into stainless steel probe tip support

Peptide mixtures (as in Figure 1 with dihydroxybenzoic acid in 30% methanol/0.1% TFA) on all four surfaces. FIGURE 4

Affinity-directed laser desorption (with matrix added as described in prior art)

Examples:

Group 1. Immobilized metal ion as the affinity ligand

Cu(II) was chelated by iminodiacetate group covalently attached to either porous agarose beads (Chelating Sepharose Fast Flow, Pharmacia Biotech Inc., Piscataway, NJ, ligand density 22-30 umole/ml gel) or solid TSK-SW beads (ToyoSoda, Japan, ligand density 20 umole/ml gel)

Fe(III) was chelated by tris(carboxymethyl)ethylenediamine-Sepharose 6B (synthesized as described by Yip and Hutchens, Protein Expression and Purification 2(1991)355-362, ligand density 65 umole/ml)

1. A mixture of synthetic peptides, neurotensin (30 nmole), sperm activating peptide (50 nmole) and angiotensin I (150 nmole), were mixed with 50 ul packed volume of TSK SW-IDA-Cu(II) at pH 7.0 (20 mM sodium phosphate, 0.5 M NaCl) at room temperature for 10 min. The gel was then washed with 3 x 200 ul sodium phosphate buffer, containing 0.5 M NaCl, pH 7.0 and suspended in equal volume of water. 2 ul of the gel suspension was mixed with 1 ul sinapinic acid (methanol). FIGURE 5A showed the molecular ions (and multiple Na-adducts) of neurotensin and sperm activating factor which were not adsorbed by the IDA-Cu(II). The mass spectrum in FIGURE 5B showed mainly the angiotensin I plus Na-adducts. When the IDA-Cu(II) gel was further washed with 500 ul of water 2x, the resulting mass spectrum showed only the parent angiotensin I species with no other adduct peaks. When the IDA-Cu(II) gel beads with adsorbed angiotensin was incubated with cyanohydroxycinnamic acid (20 mg/ml water) pH 7.0 for 10 min at room temperature and then analyzed separately, the angiotensin I was found to be still associated with the gel beads and not with the matrix solution.

2. Horse heart myoglobin (325 pmole) was mixed with 50 ul of TSK SW-IDA-Cu(II) gel beads in 20 mM sodium phosphate, 0.5 M NaCl, pH 7.0 at room temperature for 10 min. The gel beads were then washed with 2 x 500 ul of buffer and 2 x 500 ul of

water. The beads were suspended in equal volume of water and then serial diluted into water. 0.5 ul of the diluted gel suspension was mixed with 1 ul of sinapinic acid (30% methanol/0.1% TFA). A detectable signal (after averaging 50 laser shots) of myoglobin was still obtained when the calculated quantity equivalent to or less than 8 fmole was deposited on the probe tip. FIGURE 6

3. 100 ul of infant formula and gastric content of preterm infant aspirated 90 min after feeding of infant formula was mixed with 50 ul of TED-Fe(III) in 0.1 M MES, 0.15 M NaCl, pH 6.5 at room temperature for 15 min. The gel beads were then washed with 3 x 500 ul of MES buffer and then with 1 x 500 ul of water. 1 ul of the gel suspension was mixed with 2 ul of sinapinic acid (50% acetonitrile/0.1% TFA). The result showed that gastric aspirate had much more low molecular weight phosphopeptides (i.e., bound by TED-Fe(III)) than the formula due to proteolytic digestion. *In situ* alkaline phosphatase digestion of peptides adsorbed on the TED-Fe(III) gel beads showed shifts to lower molecular weight indicating that they are indeed phosphopeptides. FIGURE 7

Group 2. Immobilized antibody as the affinity ligand

Polyclonal rabbit anti-human lactoferrin antibody was custom generated for this lab by Bethyl Laboratories (Montgomery, TX). It was purified by thiophilic adsorption and then by immobilized lactoferrin column. Sheep anti-rabbit IgG covalently attached to magnetic beads were obtained from Dynal AS (Oslo, Norway) (uniform

2.8 um superparamagnetic polystyrene beads, ligand density 10 ug sheep IgG per mg bead).

1. Human lactoferrin (1 nmole) was incubated with rabbit antihuman lactoferrin at 37° for 30 min. Subsequently, 40 ul of sheep anti-rabbit IgG on Dynabeads (6-7 x $10^8$ beads/ml) was added and incubated at 37° for 30 min. The beads were then washed with 3 x 500 ul of sodium phosphate buffer, and 2 x 500 ul of water. The final amount of human lactoferrin bound to the complex was estimated to be 4 pmole. Approximately 1/10 of the beads was transferred to a magnetic probe tip and mixed with 2 ul of sinapinic acid (30% MeOH/0.1% TFA). Result showed the lactoferrin ion signal in addition to the rabbit IgG signal. FIGURE 8

Group 3. Immobilized nucleic acid as the affinity ligand

Single-strand DNA immobilized on 4% agarose beads was obtained from GIBCO BRL, Gaithersburg, MD. The ligand density was 0.5-1.0 mg/ml.

1. 200 ul of $^{125}$I human lactoferrin (equivalent to 49 nmole) was mixed with 100 ul of immobilized single-strand DNA in 20 mM HEPES, pH 7.0 at room temperature for 10 min. The beads were then washed with 5 x 500 ul of HEPES buffer and then suspended in equal volume of water. The amount of lactoferrin bound per bead was found to be 62 fmole by determining the radioactivity and counting the number of beads per unit volume. Various numbers of beads (from 1 to 12) were deposited on 0.5 mm diameter probe tips and mixed with 0.2 ul of sinapinic acid (30%

methanol/0.1% TFA). Lactoferrin ion signals were obtained with multiple 100 laser shots on a single bead with adsorbed lactoferrin. FIGURE 9

2. 30 pmole of $^{59}$Fe-human lactoferrin was added to 1 ml of preterm infant urine and mixed with 20 ul of immobilized single-strand DNA on agarose in 0.1 M HEPES pH 7.4 at room temperature for 15 min. The beads were washed with 2 x 500 ul HEPES buffer, and 2 x 500 ul of water. The beads were then suspended in equal volume of water and 1 ul (equivalent to not more than 350 fmole as determined by radioactivity) was mixed with 1 ul sinapinic acid (30% methanol/0.1% TFA) on a probe tip. Positive lactoferrin signals were obtained for multiple 50 laser shots. FIGURE 10

Group 4. Immobilized biomolecule as the affinity ligand

Soybean trypsin inhibitor (Sigma, St Louis, MO) was immobilized on AffiGel 10 (BioRad Laboratories, Hercules, CA, ligand density 15 umole/ml) according to manufacturer's instructions.

1. 100 ul of human gastrointestinal aspirate was mixed with 50 ul of immobilized soybean trypsin inhibitor in 20 mM sodium phosphate, 0.5 M sodium chloride, pH 7, at room temperature for 15 min. The gel beads were then washed with 3 x 500 ul of phosphate buffer, and 2 x 500 ul of water. 1 ul of gel bead suspension was

mixed with 2 ul of sinapinic acid (50% acetonitrile/0.1% TFA). Result showed the presence of trypsin and trypsinogen in the aspirate.

FIGURE 11

Group 5. Immobilized dye as the affinity ligand

Cibacron Blue 3GA-agarose (Type 3000, 4% beaded agarose, ligand density 2-5 umoles/ml, Sigma).

Other immobilized dyes include Reactive Red 120-agarose, Reactive Blue-agarose, Reactive Green-agarose, Reactive Yellow-agarose (Sigma)

1. 200 ul of human plasma was mixed with 50 ul of immobilized dye in 20 mM sodium phosphate, 0.5 M NaCl, pH 7.0 at room temperature for 10 min. The gel beads were then washed with 3 x 500 ul of phosphate buffer and 2 x 500 ul of water. 1 ul of gel bead suspension was mixed with 2 ul of sinapinic acid (50% acetonitrile/0.1% TFA). Result showed the selective adsorption of human serum albumin from the serum sample by Cibacron Blue. FIGURE 12

Surface-enhanced laser desorption

Examples:

Group 1. Energy-absorbing molecule covalently bonded to surface via the N-group

Cinnamamide (Aldrich, not a matrix by prior art) was dissolved in isopropanol/0.5 M sodium carbonate (3:1) and mixed with divinyl sulfone (Fluka, Ronkonkoma, NY)

activated Sepharose (Pharmacia) at room temperature for 2 hr. The excess molecules were washed away with isopropanol. The proposed structure was presented in FIGURE 13. 2 ul of bound or free molecule was deposited on the probe tips, 1 ul of peptide mixtures in 0.1% TFA was added on top and the result showed the peptide ion signals detected only for the bound form. FIGURE 14.

Group 2. Energy absorbing molecule covalently bonded to surface via the C-group

Cynnamyl bromide (Aldrich, not a matrix by prior art) was dissolved in isopropanol/0.5 M sodium carbonate and mixed with divinyl sulfone-activated Sepharose at room temperature overnight. The excess molecules were washed away with isopropanol. The proposed structures are presented in FIGURE 15. 2 ul of the bound or free molecule was deposited on the probe tip, 1 ul of peptide mixtures in 0.1% TFA was added on top and the result showed the detection of peptide ion signal only for the bound form. FIGURE 16.

Group 3. Energy absorbing molecule covalently bonded to surface via the C-group

Dihydroxybenzoic acid was activated by carbodiimide and mixed with Fmoc-MAP 8 branch resin (Applied Biosystems, Forster City, CA) overnight. The proposed structure was presented in FIGURE 17. After washing, 1 ul of the bonded molecule on MAP or the MAP alone in 50% acetonitrile/0.1% TFA were deposited on the

probe tip, 1 ul of peptide mixture was added on top, the resulting mass spectrum was presented in FIGURE 21.

Group 4. Energy absorbing molecule covalently bonded to surface via undetermined group.

Cyanohydroxycinnamic acid was dissolved in methanol and mixed with AffiGel 10 (BioRad) at room temperature for two hours. The unbound molecules were washed away with methanol. Protein samples that are found to desorb successfully from this modified surface include myoglobin (FIGURE 19), trypsin and carbonic anhydrase.

These examples (Groups 1-4) are also demonstrations of combined surface-enhanced and affinity-directed desorption where the adsorbed (bonded) energy absorbing molecular also act as affinity adsorption reagents to enhance the capture of analyte molecules.

Definitions:

(1) "Presenting surface" - the probe tip, sample plate or other surface on which the analyte and matrix are presented for desorption/ionization and analysis for example by mass spectrometry.

(2) "Matrix" - as described in prior art as the substance mixed with the analyte (typically prior to deposition) and deposited on the presenting surface in association with the analyte to absorb at least part of the energy from the

energy source (e.g., laser) to facilitate desorption of intact molecules of the analyte.

(3) "Analyte" - the material which is the subject of desorption and investigation by mass spectrometry or other means for detection.

(4) "Affinity reagent" (analyte capture device) - the class of molecules (both man made, unnatural, natural and biological) and/or compounds which have the ability of being retained on the presenting surface (by covalent bonding, chemical absorption, etc.) while retaining the ability of recognition and bonding to an analyte.

(5) "Desorption" - the departure of analyte from the surface and/or the entry of the analyte into a gaseous phase.

(6) "Ionization" - the process of creating or retaining on an analyte an electrical charge equal to plus or minus one or more electron units.

(7) "Adduct" - the appearance of an additional mass associated with the analyte and usually caused by the reaction of excess matrix (or matrix break-down products) directly with the analyte.

(8) "Adsorption" - the chemical bonding (covalent and/or noncovalent) of the energy-absorbing molecules, the affinity reagent (i.e., analyte capture device), and/or the analyte to the probe (presenting surface).

## CLAIMS

What is claimed:

1. An apparatus for measuring the mass of analyte molecules by means of mass spectrometry, said apparatus comprising:

a spectrometer tube;

vacuum means for applying a vacuum to the interior of said tube;

electrical potential means within the tube for applying an accelerating electrical potential to desorbed analyte molecules;

sample presenting means removably insertable into said spectrometer means, for presenting said analyte molecules in association with a matrix material for promoting desorption and ionization of said analyte molecules, said sample presenting means being adapted to present said analyte molecules on or above the surface of said matrix material, whereby at least a portion of said analyte molecules not consumed in said mass spectrometry analysis will remain accessible for subsequent chemical analytical procedures;

laser beam means for producing a laser beam directed to said analyte molecules and matrix material on said sample presenting means inserted into said spectrometer means, for imparting sufficient energy to desorb and ionize a portion of said analyte molecules on said sample presenting means; and

detector means associated with said spectrometer tube for detecting the impact of accelerated ionized analyte molecules thereon.

2. A method in mass spectrometry to measure the mass of an analyte molecule, said method comprising the steps of:

derivitizing a surface on a probe tip face with an affinity reagent having means for selectively bonding with an analyte molecule;

exposing said derivitized probe tip face to a source of said analyte molecule so as to bond said analyte molecule thereto;

placing the probe tip into one end of a time-of-flight mass spectrometer and applying a vacuum and an electric field to form an accelerating potential within the spectrometer;

striking the probe tip face within the spectrometer with a series of laser pulses in order to desorb ions of said analyte molecules from said tip;

detecting the mass weights of the ions by their time of flight within said mass spectrometer; and

displaying such detected mass weights.

3. The method according to claim 2 comprising additionally applying a desorption assisting matrix material to said probe tip face in association with said affinity reagent, said matrix material being applied in a manner so as not to interfere with said means on said affinity reagent for selectively bonding with said analyte molecules,

a portion of said analyte molecules which are not desorbed from said probe tip remaining chemically accessible for subsequent analytical procedures without the necessity for separating them from said matrix material.

4. The method according to claim 3 comprising additionally,

removing said probe tip from said mass spectrometer;

performing a chemical procedure on said portion of said analyte molecules so as to alter the chemical composition of said portion of said analyte molecules;

reinserting said probe tip with said chemically altered analyte molecules thereon; and

performing a subsequent mass spectrometry analysis to determine the molecular weight of said chemically altered analyte molecules.

5. The method according to claim 2 wherein said affinity reagent is chemically bonded to said face of said probe tip.

6. The method according to claim 2 wherein said affinity reagent is physically adhered to said face of said probe tip.

7. The method according to claim 2 wherein said affinity reagent is adapted to chemically bond to said analyte molecules.

8. The method according to claim 2 wherein said affinity reagent is adapted to biologically adhere to said analyte molecules.

9. The method according to claim 2 wherein said analyte molecules are biomolecules and said affinity reagent is adapted to selectively isolate said biomolecules from an undifferentiated biological sample.

10. The method according to claim 3 wherein said matrix materials are in the weakly acidic to strongly basic pH range.

11. The method according to claim 3 wherein said matrix materials have a pH above 6.0.

12. The method according to claim 2 wherein said face of said probe tip is formed of an electrically insulating material.

13. A method of measuring the mass of analyte molecules by means of laser desorption/ionization, time-of-flight mass spectrometry in which a matrix material is used in conjunction with said analyte molecules for facilitating desorption and ionization of the analyte molecules, the improvement comprising:

presenting the analyte molecules on or above the surface of the matrix material, whereby at least a portion of the analyte molecules not desorbed in said mass spectrometry analysis remain chemically accessible for subsequent analytical procedures, *in situ*, on said probe tip, without the necessity for separating said portion of said analyte molecules from said matrix material.

14. An apparatus for facilitating desorption and ionization of analyte molecules for analysis by mass spectrometry, said apparatus comprising:

a substrate; and

an affinity reagent attached to said substrate and having means for selectively bonding with said analyte molecules.

15. The apparatus according to claim 14 wherein said substrate comprises the surface of a probe tip for use in a time-of-flight mass spectrometry analyzer.

16. The apparatus according to claim 14 wherein said affinity reagent is chemically bonded to said substrate.

17. The apparatus according to claim 14 wherein said affinity reagent is physically adhered to said substrate.

18. The apparatus according to claim 14 wherein said affinity reagent is adapted to chemically bond to said analyte molecules.

19. The apparatus according to claim 14 wherein said affinity reagent is adapted to biologically adhere to said analyte molecules.

20. The apparatus according to claim 14 wherein said analyte molecules are biomolecules and said affinity reagent is adapted to selectively isolate said biomolecules from an undifferentiated biological sample.

21. The apparatus according to claim 14 comprising additionally a matrix material deposited on said substrate in association with said affinity reagent in a manner so as to not render ineffective said means on said affinity reagents for selective bonding with said analyte molecules.

22. The apparatus according to claim 21 wherein said matrix material is in the weakly acidic to strongly basic pH range.

23. The apparatus according to claim 21 wherein said matrix material has a pH above 6.0.

24. The apparatus according to claim 14 wherein said substrate is formed of an electrically insulating material.

25. A method for preparing a surface for presenting analyte molecules for analysis by time-of-flight mass spectrometry, said method comprising:

providing a substrate on said surface for supporting said analyte;

derivitizing said substrate with an affinity reagent having means for selectively bonding with said analyte; and

depositing a desorption/ionization promoting matrix material on said substrate in association with said affinity reagent, said matrix material being deposited in a manner so as to not render ineffective said means on said affinity reagent for selectively bonding with said analyte.

26. A method for preparing a surface for presenting analyte molecules for analysis, said method comprising:

providing a substrate on said surface for supporting said analyte;

derivitizing said substrate with an affinity reagent having means for selectively bonding with said analyte; and

a means for detection of said analyte molecules bonded with said affinity reagent.

27. The method according to claim 26 comprising additionally the step of applying a detection material to said surface.

28. The method according to claim 27 wherein such detection material comprises a fluorescing species.

29. The method according to claim 27 wherein such detection material comprises an enzymatic species.

30. The method according to claim 27 comprising additionally wherein such detection material comprises a radioactive species.

31. The method according to claim 27 comprising additionally wherein such detection material comprises a light-emitting species.

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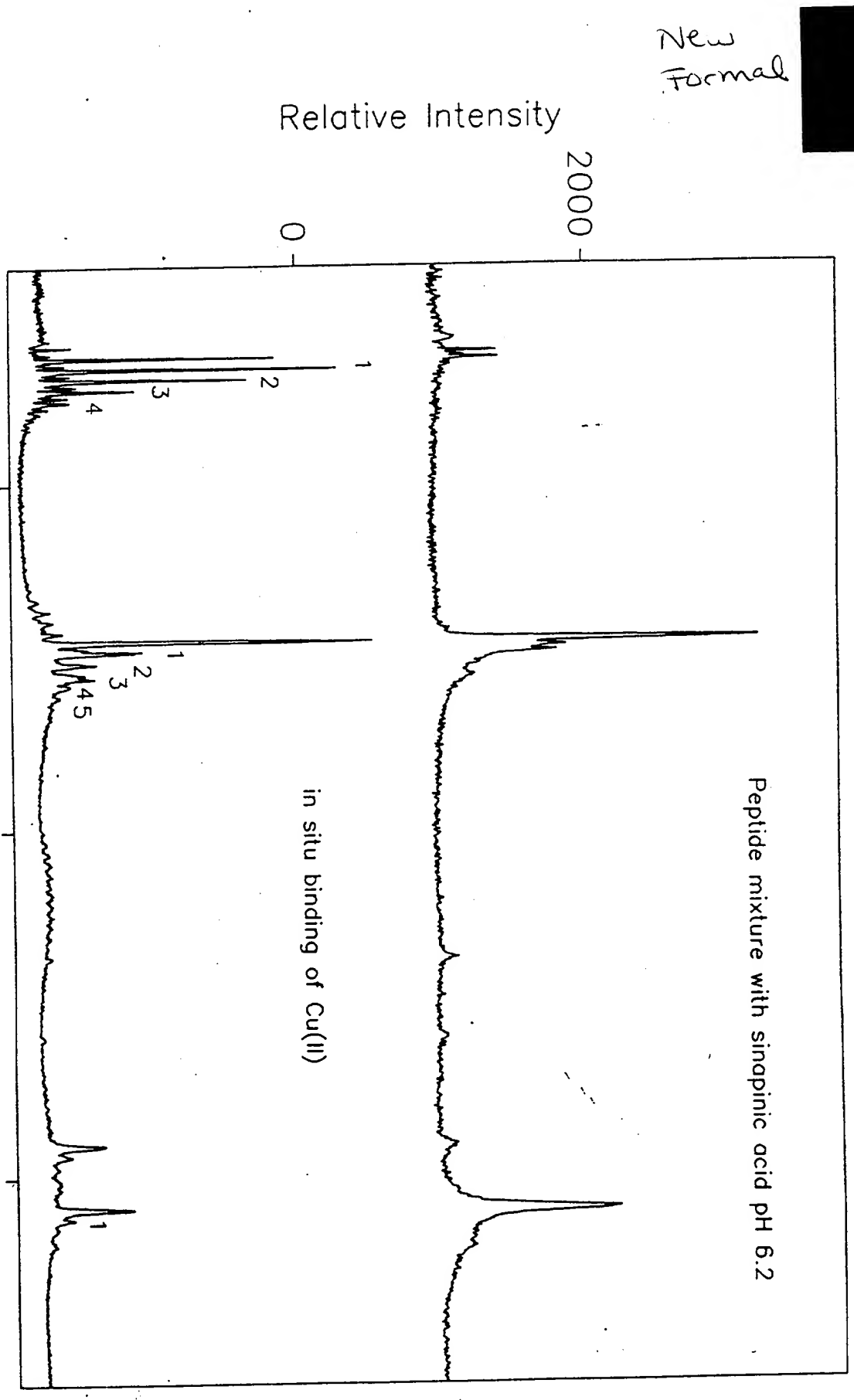

New Formal
Relative Intensity
2000
0
Peptide mixture with sinapinic acid pH 6.2
in situ binding of Cu(II)
1
2
3
4
1
2
3
4
5
1
2000
4000
6000
Mass/Charge (m/z)

FIGURE 1

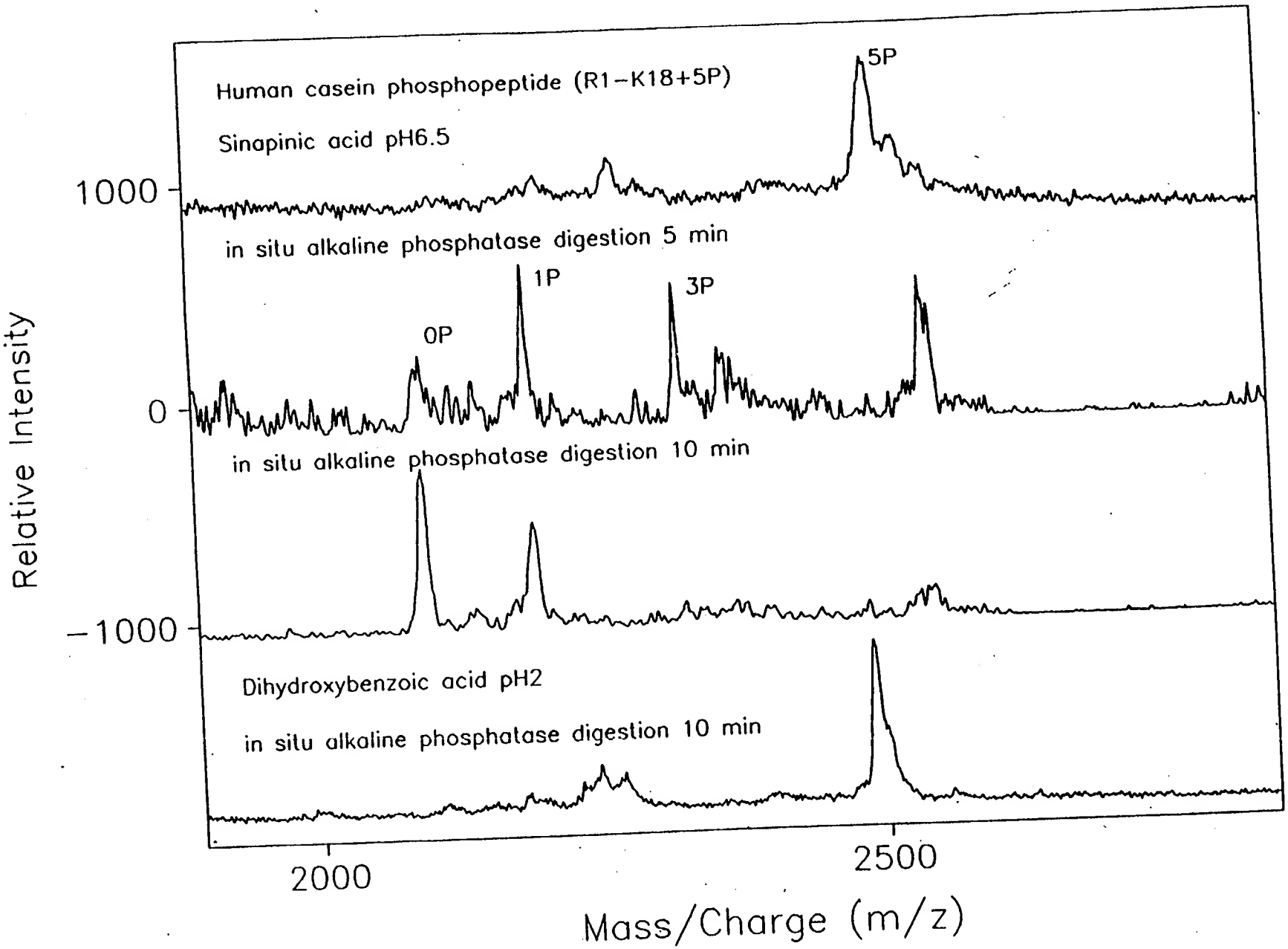

Human casein phosphopeptide (R1–K18+5P)
Sinapinic acid pH6.5
5P
1000
in situ alkaline phosphatase digestion 5 min
1P
3P
0P
0
in situ alkaline phosphatase digestion 10 min
−1000
Dihydroxybenzoic acid pH2
in situ alkaline phosphatase digestion 10 min
Relative Intensity
2000
2500
Mass/Charge (m/z)

FIGURE 2

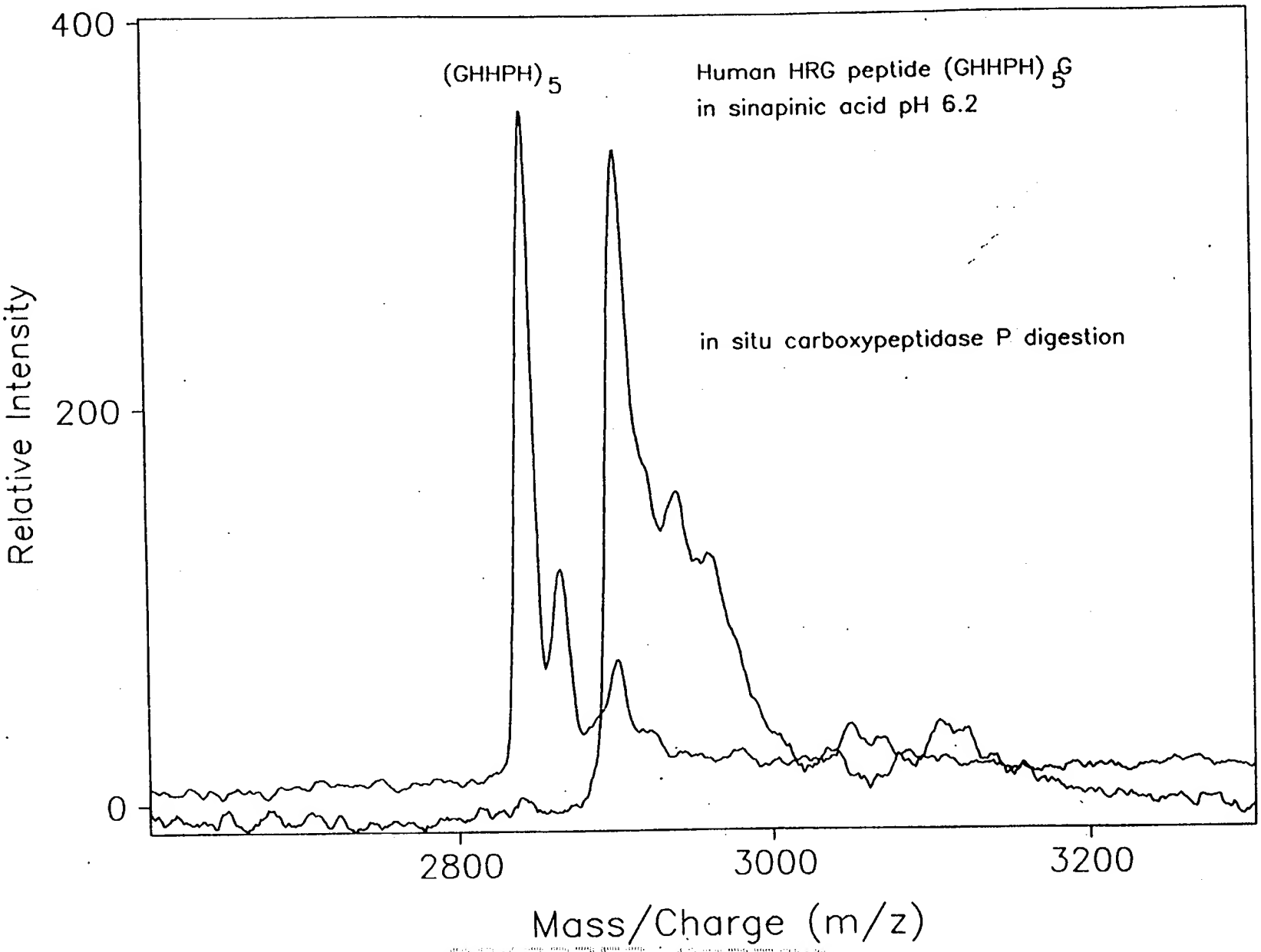

400
200
0
Relative Intensity
(GHHPH)5
Human HRG peptide (GHHPH)5G
in sinapinic acid pH 6.2
in situ carboxypeptidase P digestion
2800
3000
3200
Mass/Charge (m/z)

FIGURE 3

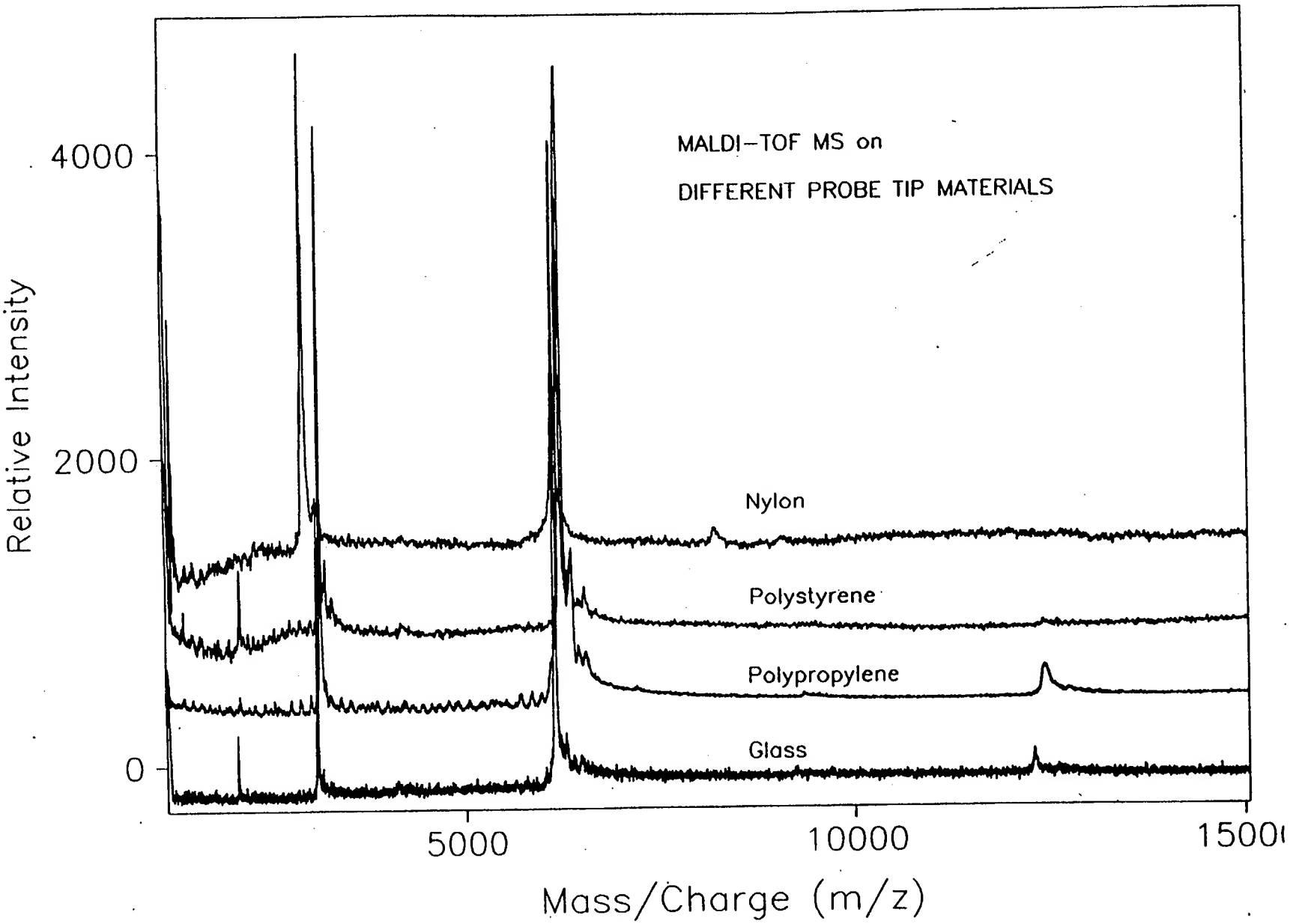

MALDI–TOF MS on
DIFFERENT PROBE TIP MATERIALS
Relative Intensity
4000
2000
0
Nylon
Polystyrene
Polypropylene
Glass
5000
10000
1500
Mass/Charge (m/z)

FIGURE 4

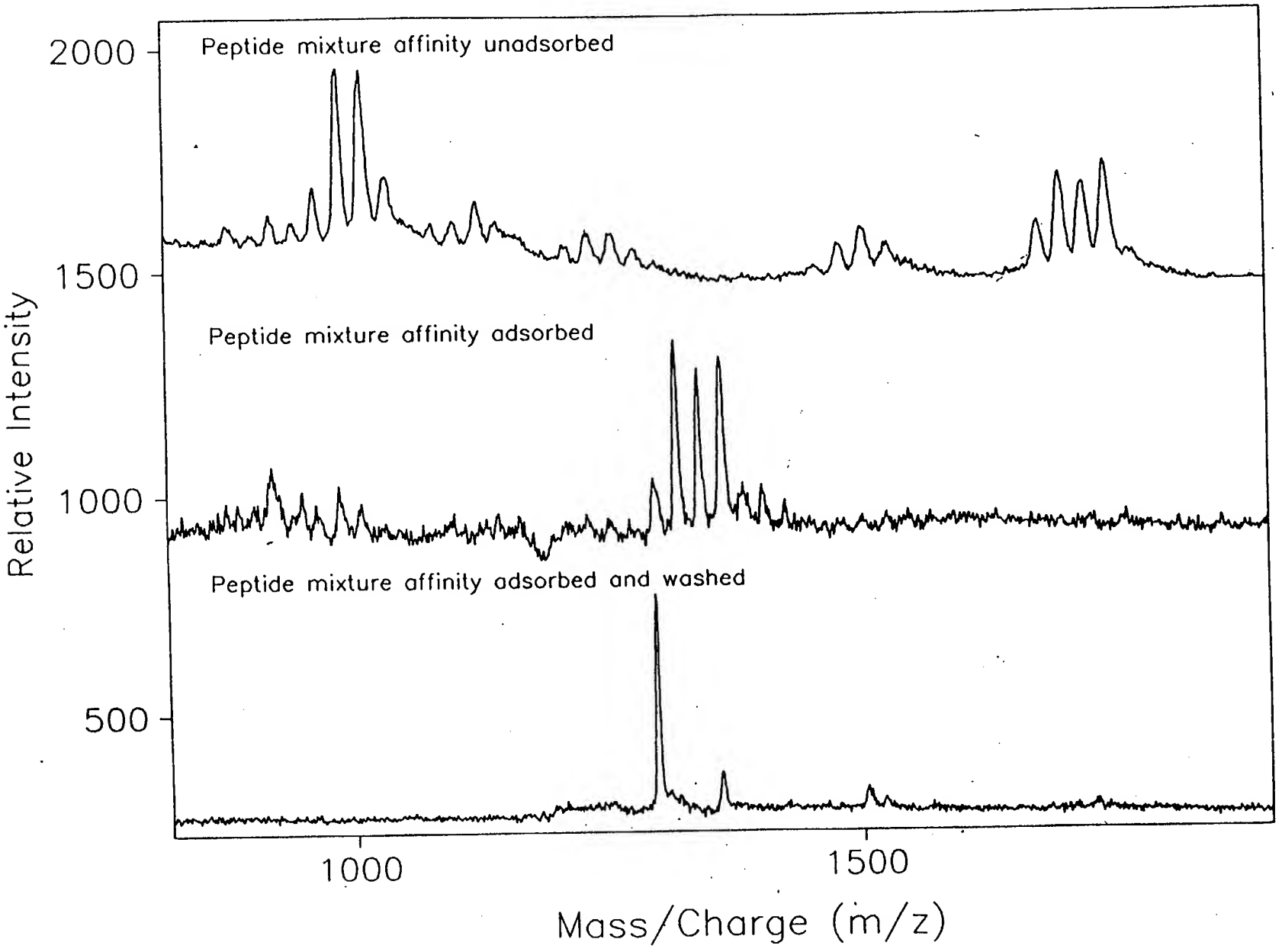
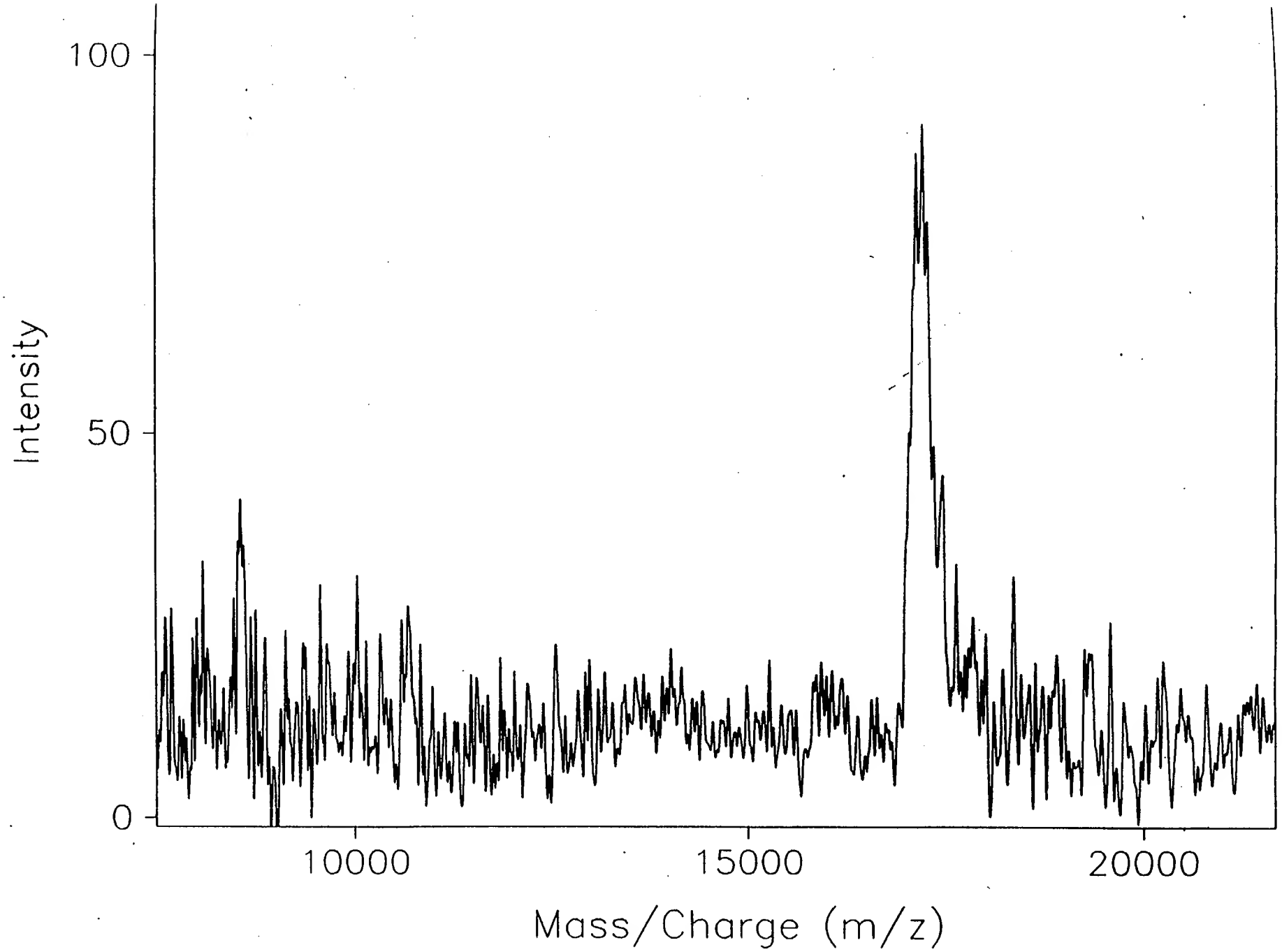

Peptide mixture affinity unadsorbed
Peptide mixture affinity adsorbed
Peptide mixture affinity adsorbed and washed
2000
1500
1000
500
Relative Intensity
1000
1500
Mass/Charge (m/z)

FIGURE 5


100
50
0
Intensity
10000
15000
20000
Mass/Charge (m/z)

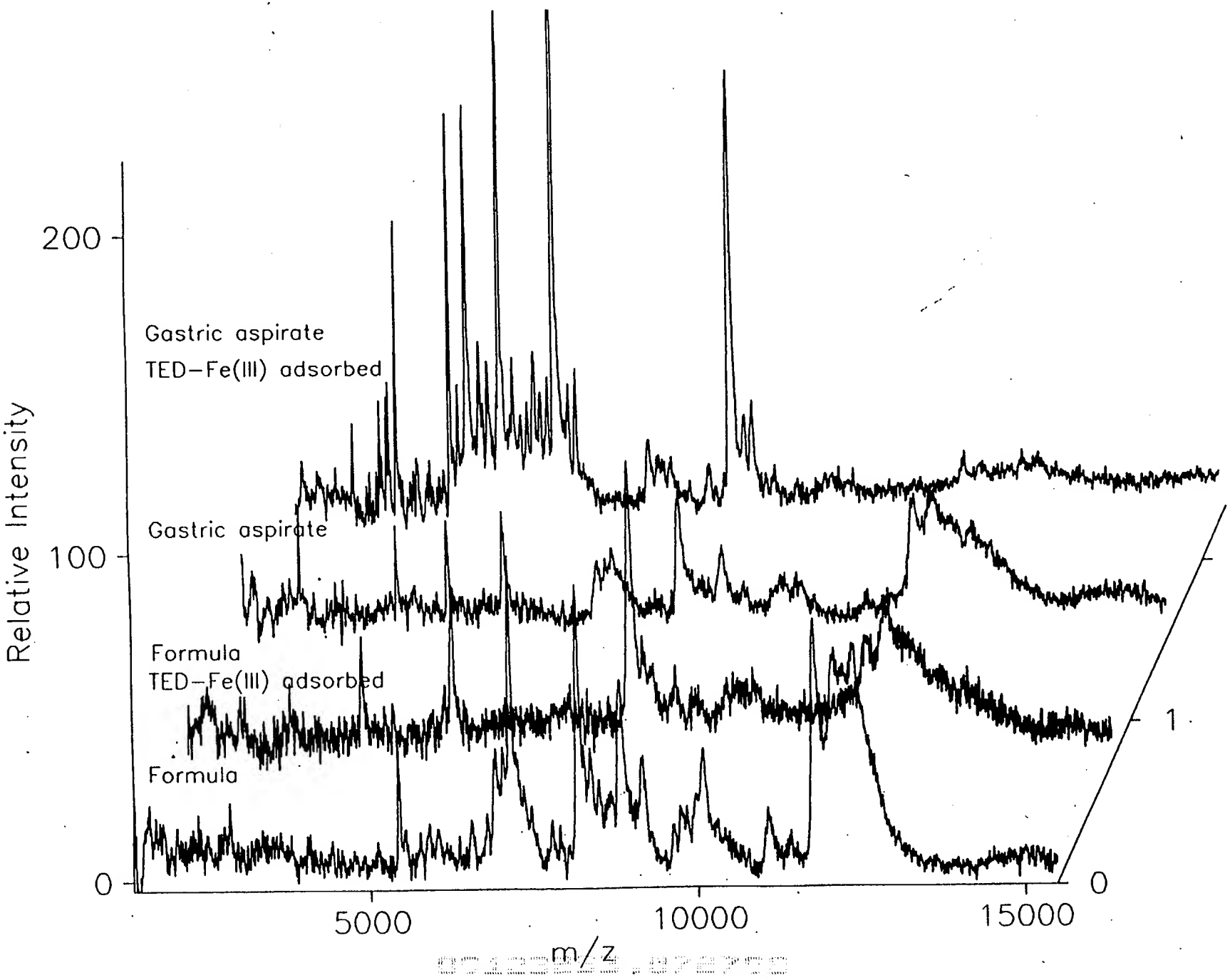
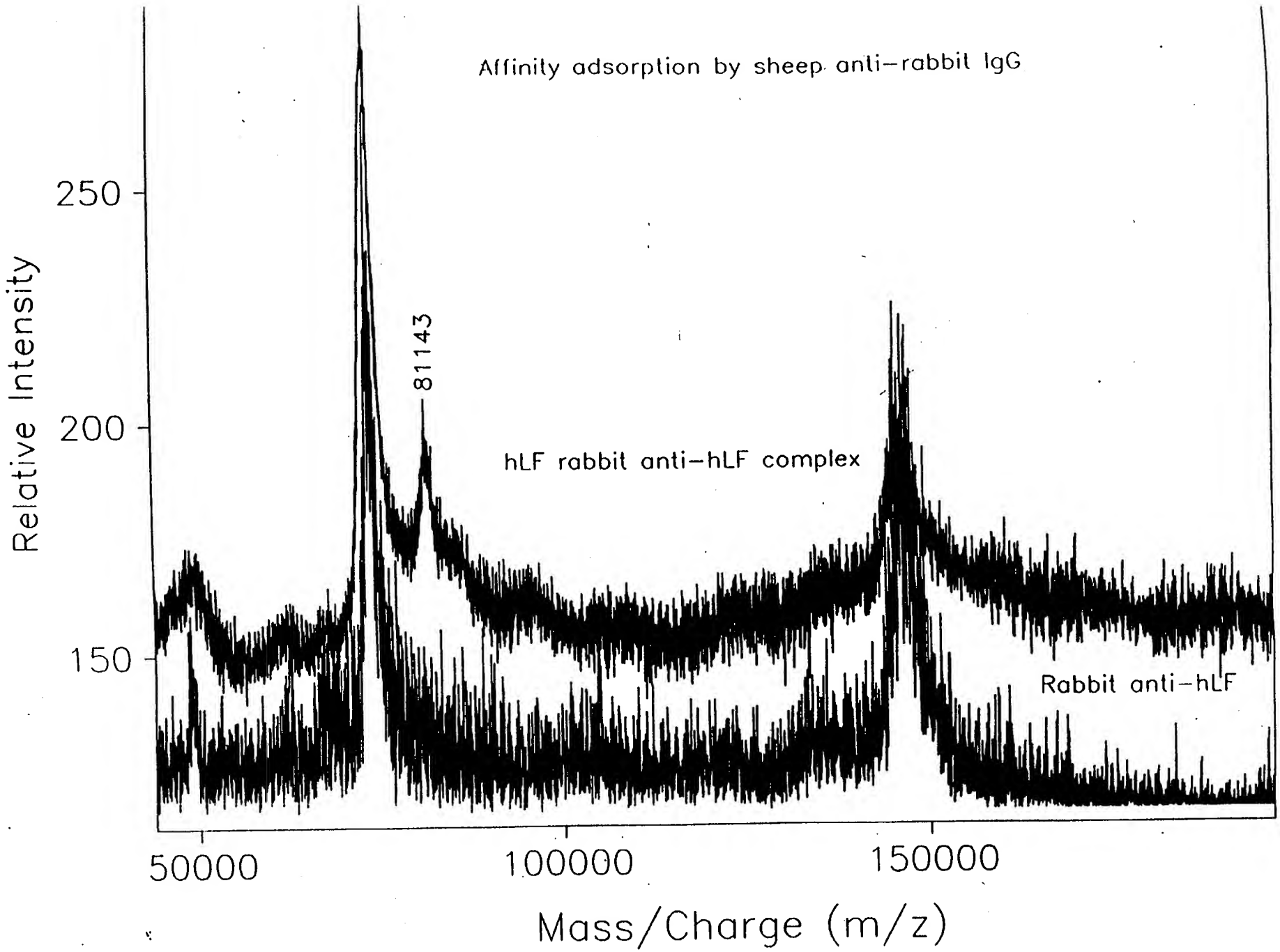

200
100
0
Relative Intensity
Gastric aspirate
TED–Fe(III) adsorbed
Gastric aspirate
Formula
TED–Fe(III) adsorbed
Formula
5000
10000
15000
m/z
1
0

FIGURE 7


Affinity adsorption by sheep anti-rabbit IgG
Relative Intensity
250
200
150
81143
hLF rabbit anti-hLF complex
Rabbit anti-hLF
50000
100000
150000
Mass/Charge (m/z)

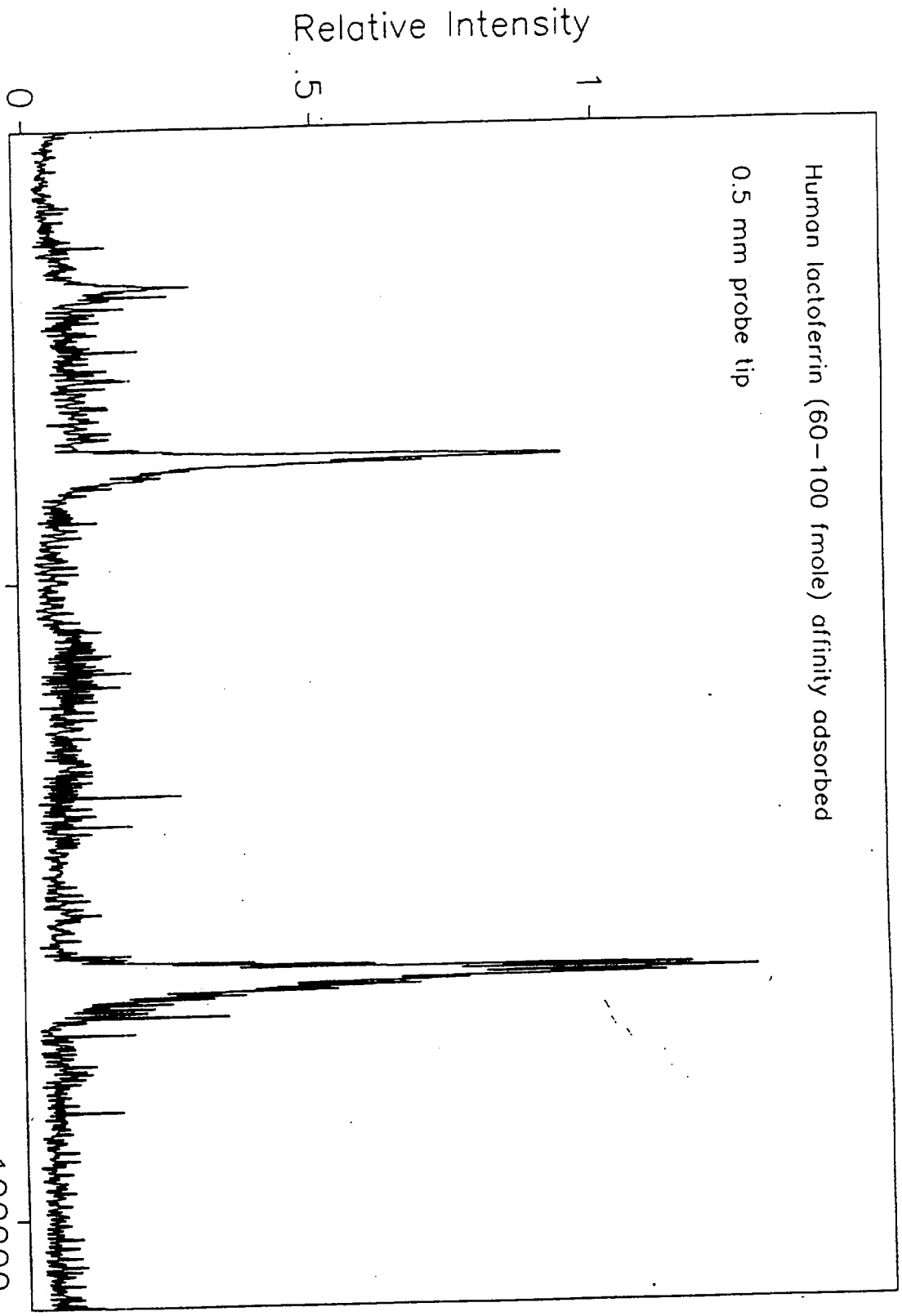

Relative Intensity
0
.5
1
Human lactoferrin (60–100 fmole) affinity adsorbed
0.5 mm probe tip
50000
100000
Mass/Charge (m/z)

FIGURE 9

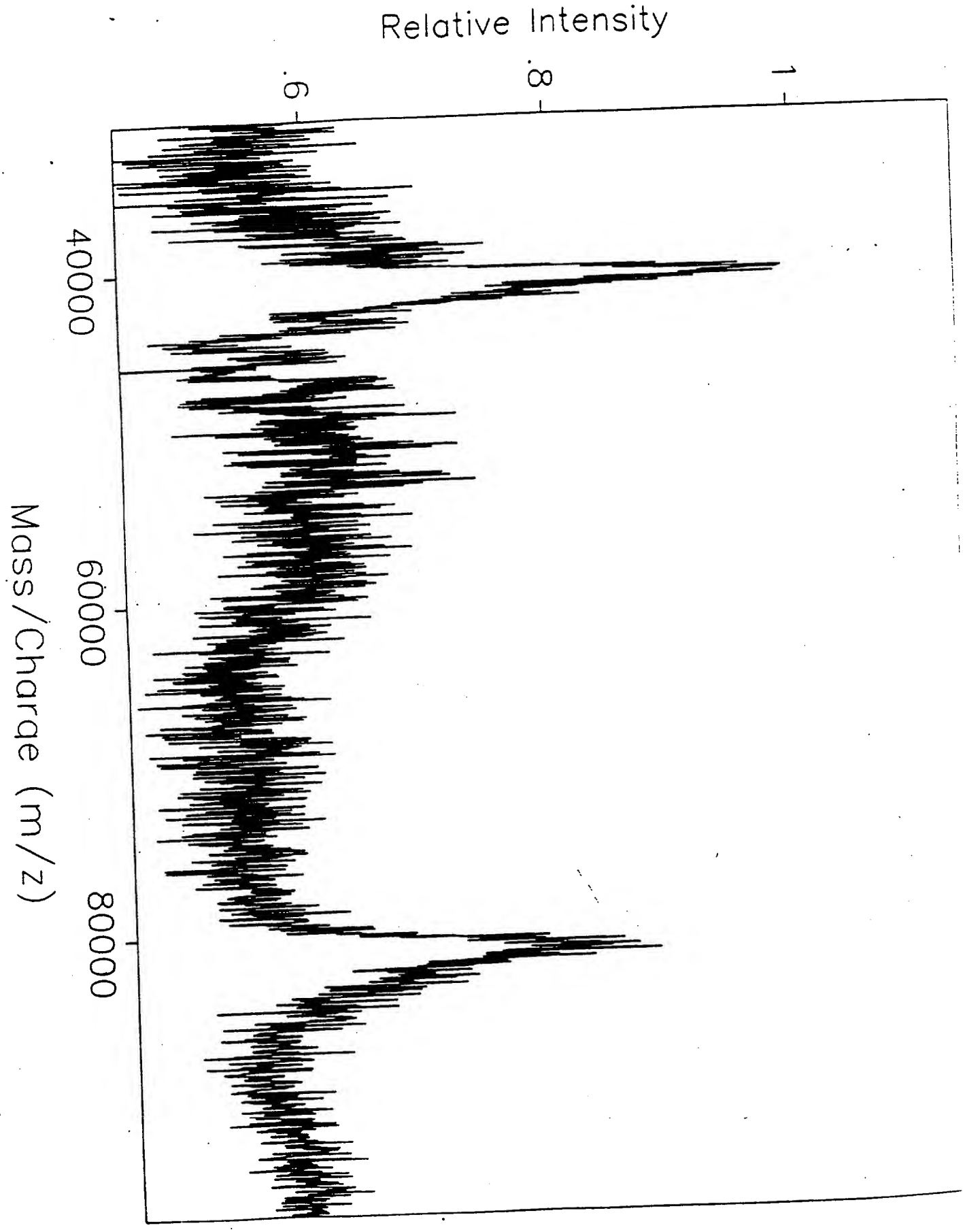
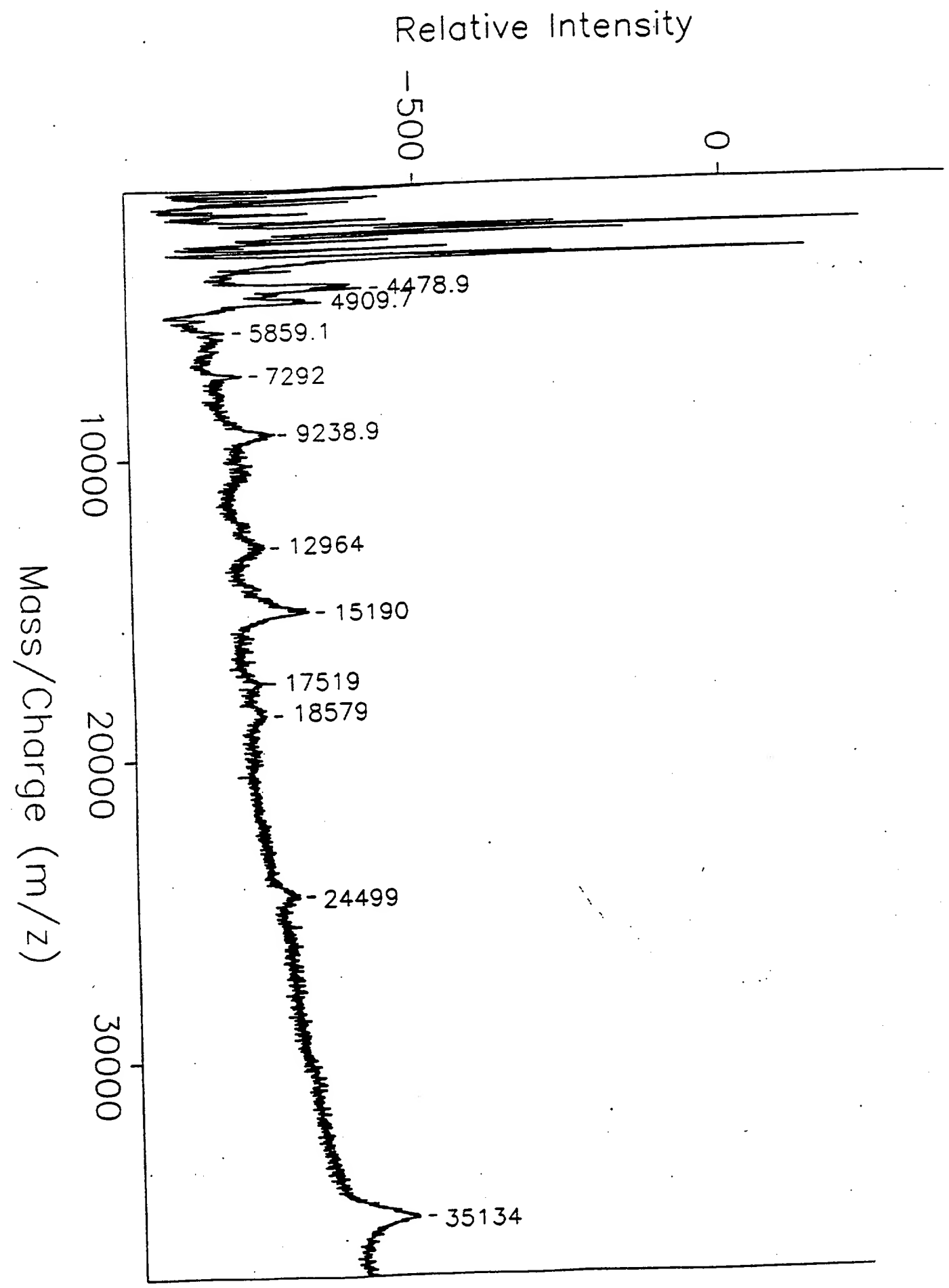



FIGURE 10


Relative Intensity
-500
0
4478.9
4909.7
5859.1
7292
9238.9
12964
15190
17519
18579
24499
35134
10000
20000
30000
Mass/Charge (m/z)

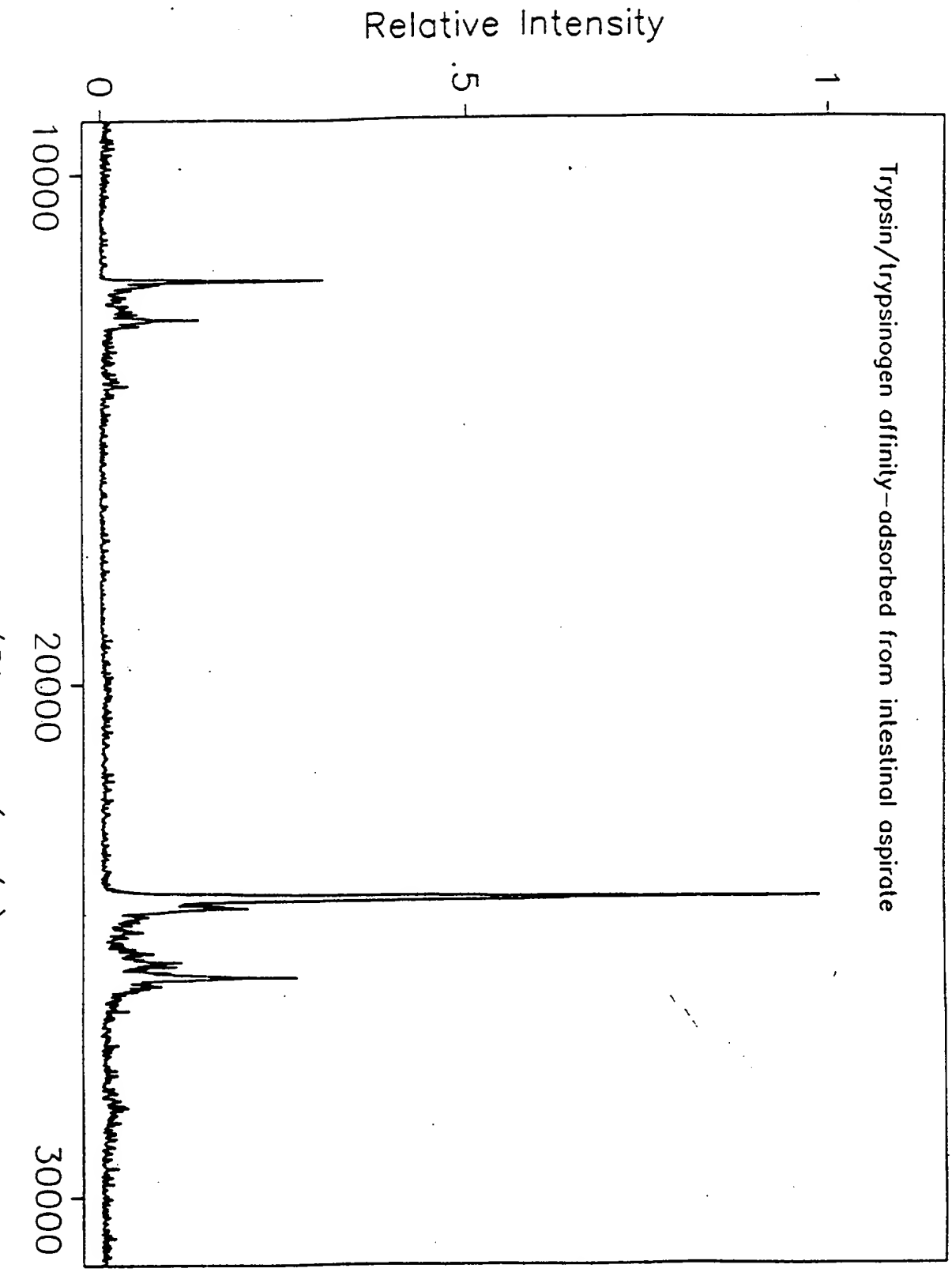
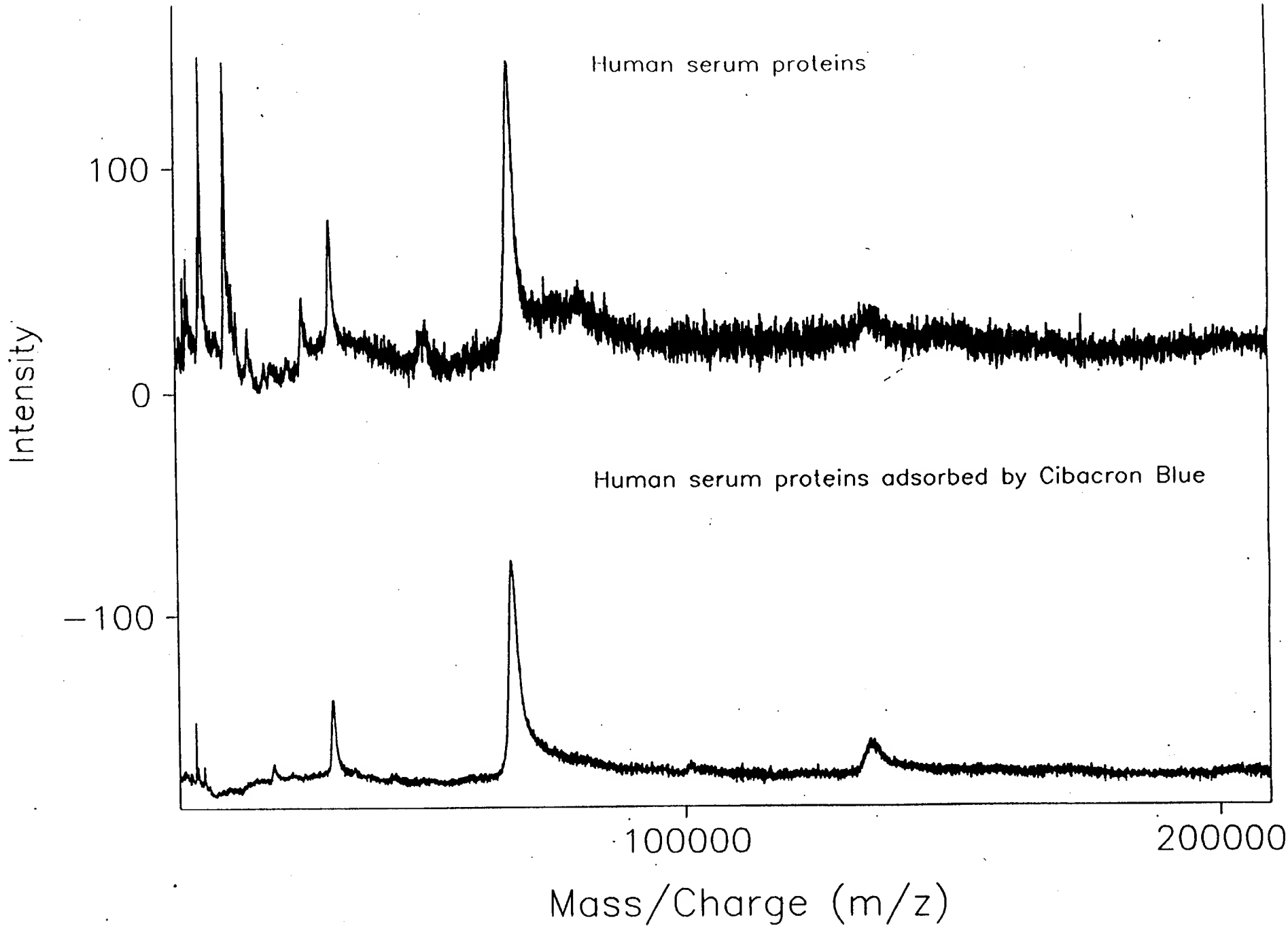

Relative Intensity
0
.5
1
Trypsin/trypsinogen affinity-adsorbed from intestinal aspirate
10000
20000
30000
Mass/Charge (m/z)

FIGURE 11 B


Human serum proteins
100
0
Intensity
Human serum proteins adsorbed by Cibacron Blue
−100
100000
200000
Mass/Charge (m/z)

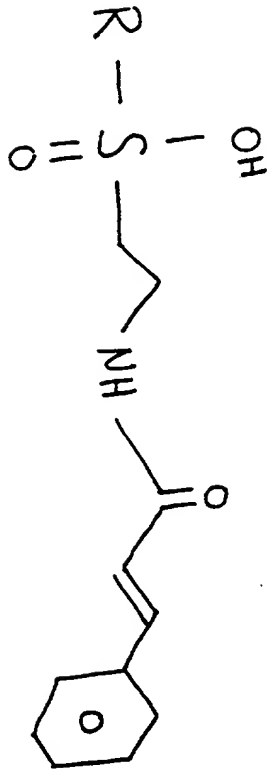



FIGURE 13

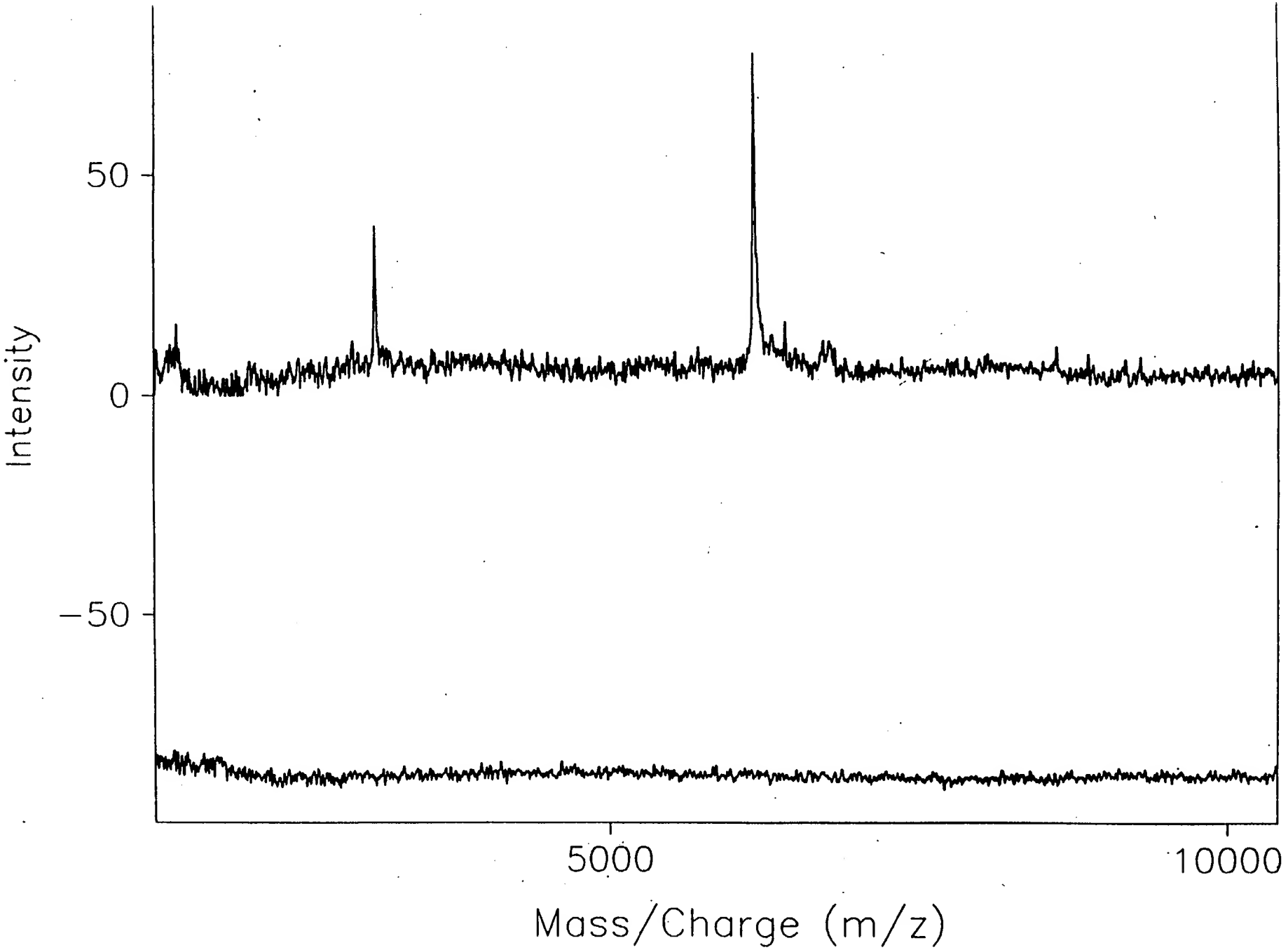



FIGURE 14

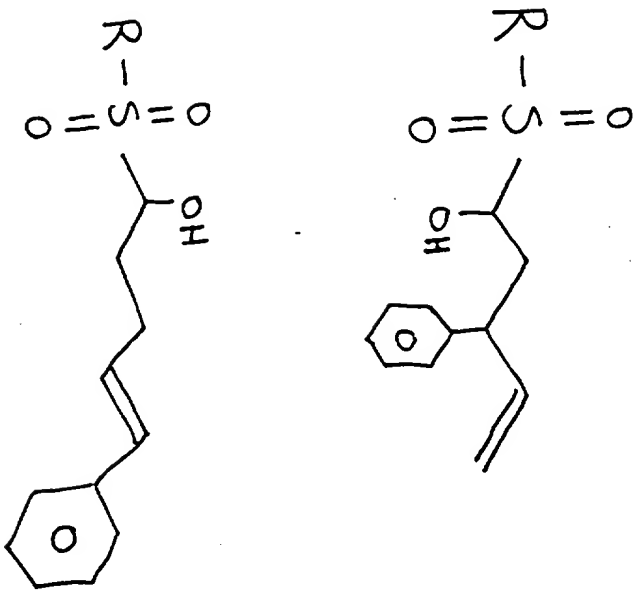


FIGURE 15

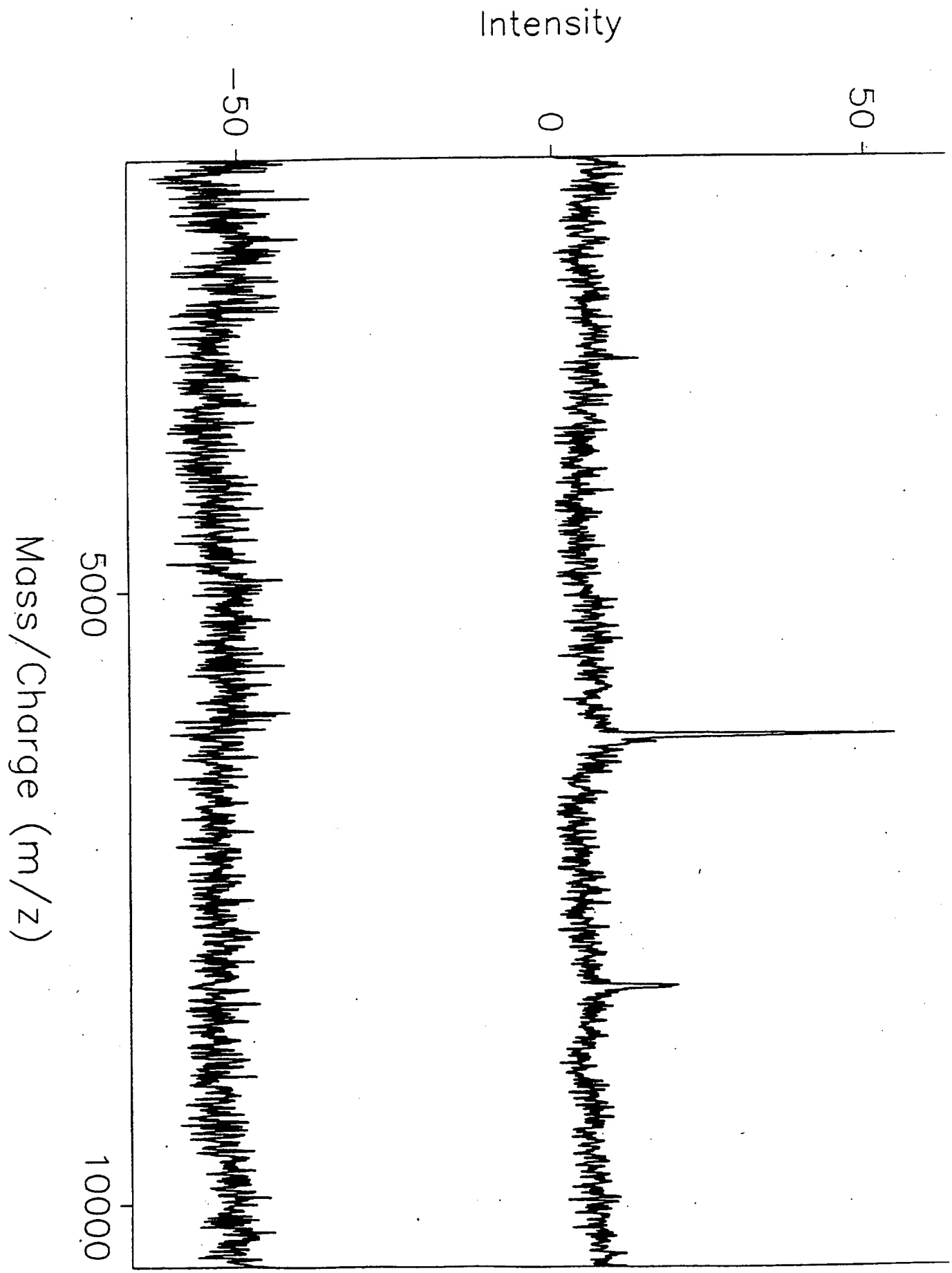



FIGURE 16

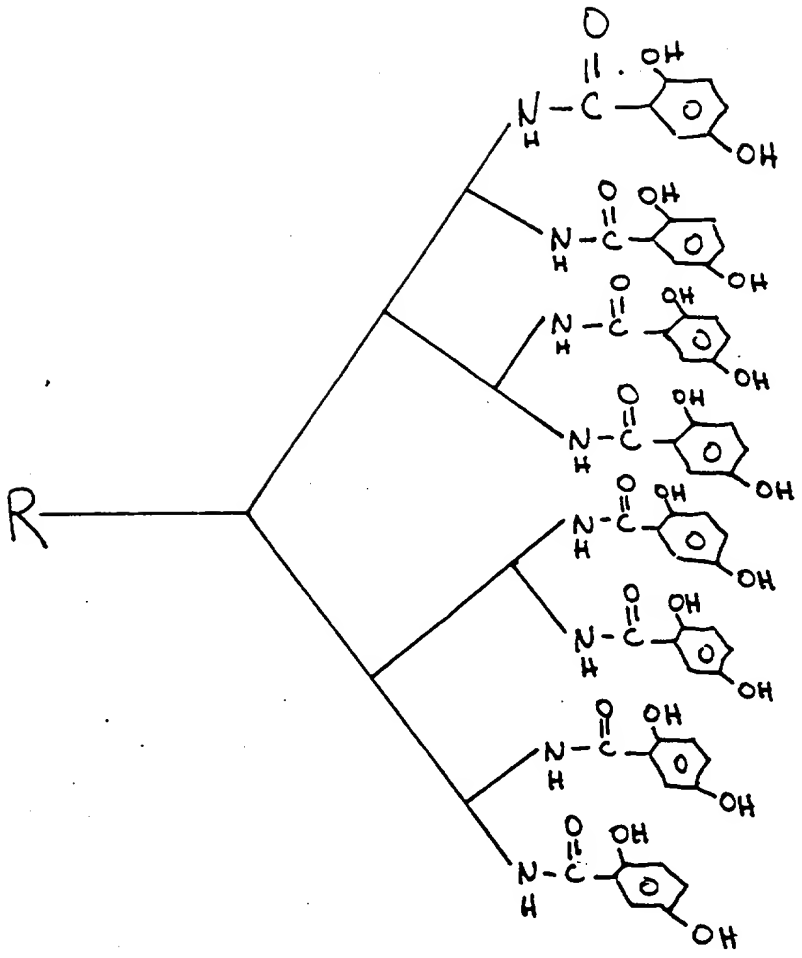



FIGURE 17

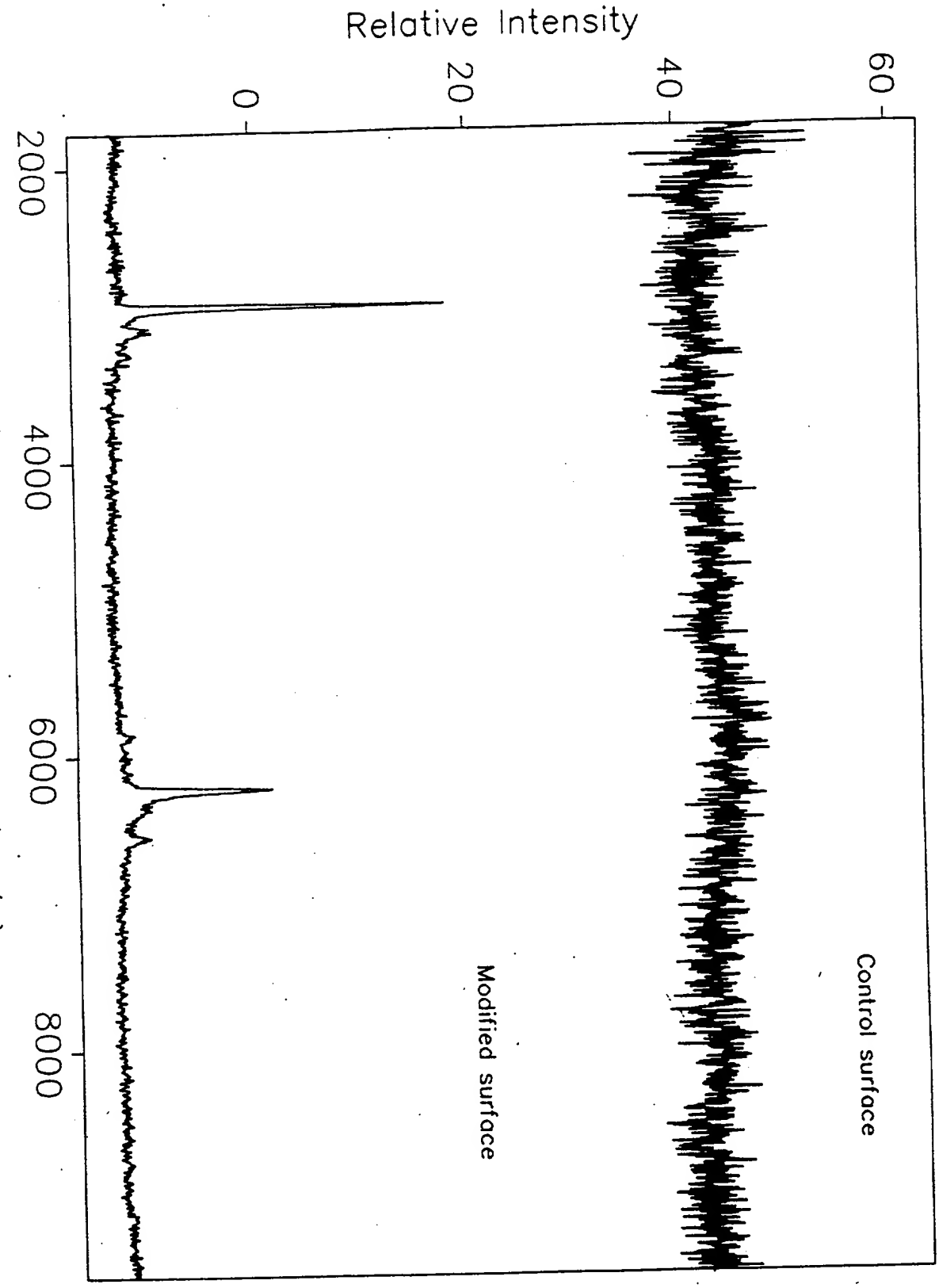
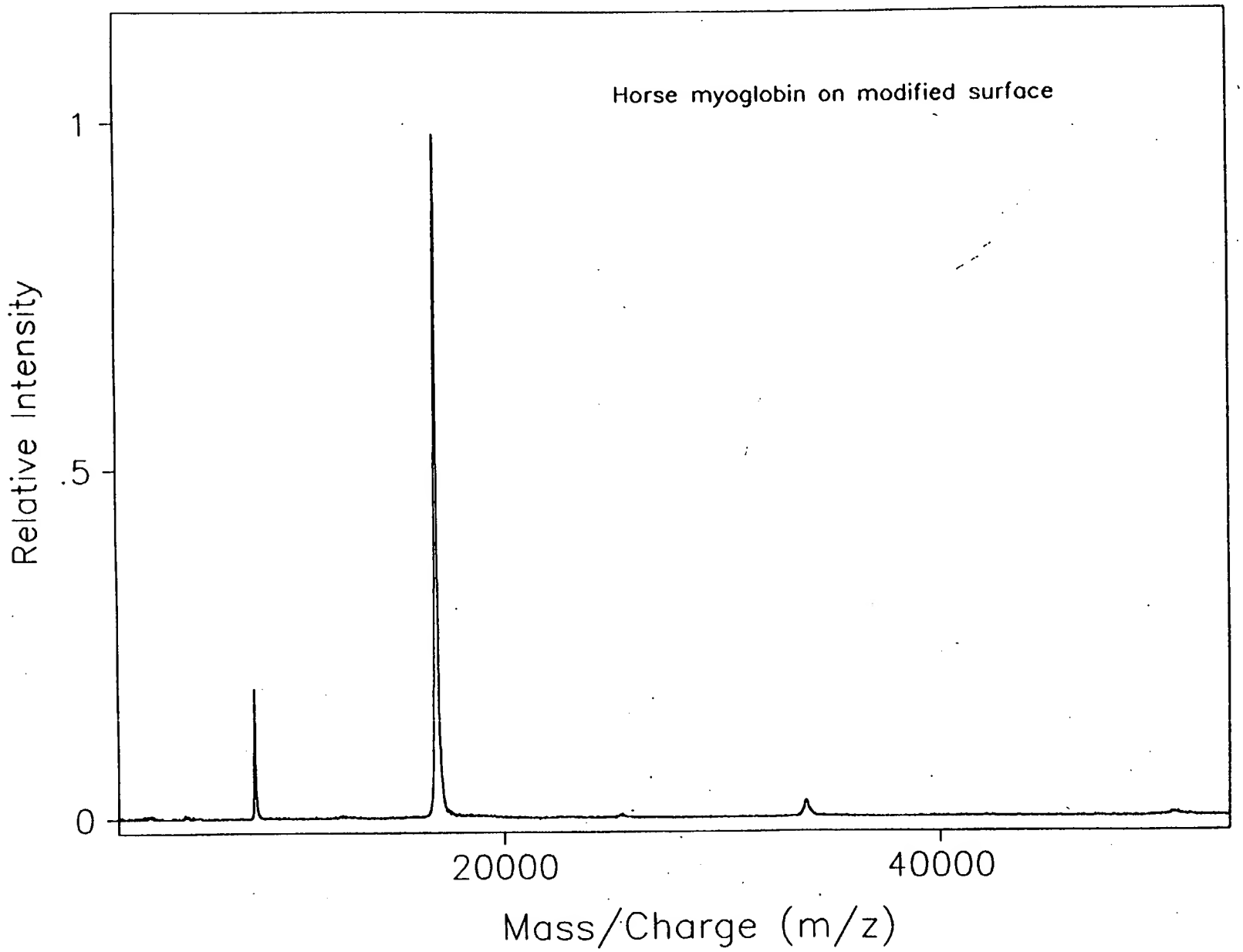

Relative Intensity
0
20
40
60
2000
4000
6000
8000
Mass/Charge (m/z)
Control surface
Modified surface

FIGURE 18


Horse myoglobin on modified surface
Relative Intensity
1
.5
0
20000
40000
Mass/Charge (m/z)

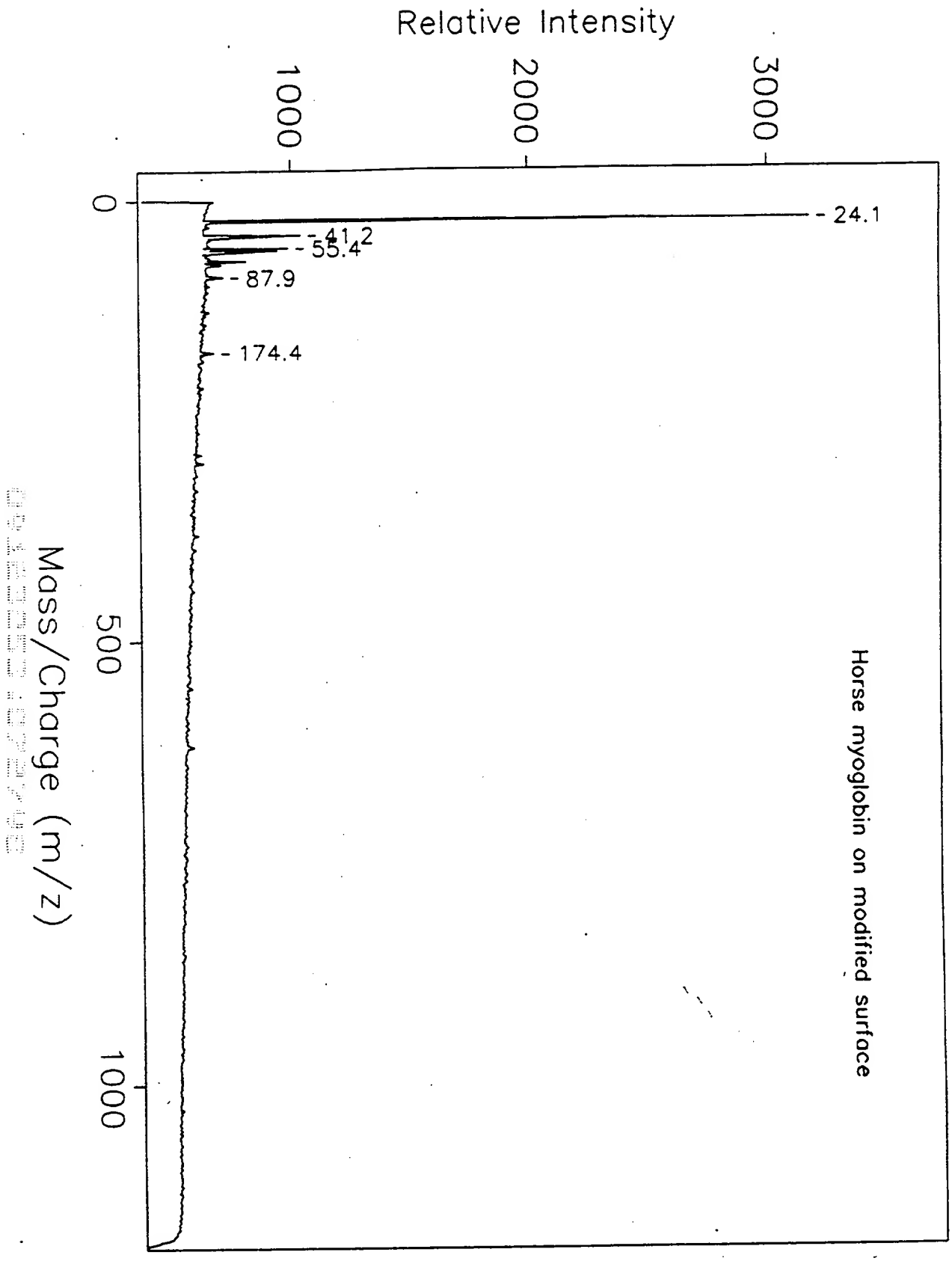

Relative Intensity
1000
2000
3000
0
500
1000
Mass/Charge (m/z)
24.1
41.2
55.4
87.9
174.4
Horse myoglobin on modified surface

FIGURE 19 B

# DECLARATION AND POWER OF ATTORNEY

As the below-named inventors, we hereby declare that:

Our residences, post office addresses and citizenship are as stated below next to our respective names;

We verily believe we are the original, first inventors of the invention entitled: METHODS AND APPARATUS FOR DESORPTION AND IONIZATION OF MOLECULES described and claimed in the attached specification;

We hereby state that we have reviewed and understand the contents of the attached specification, including the claims.

We acknowledge our duty to disclose information which is material to the examination of the application in accordance with §1.56(a), Title 37, Code of Federal Regulations.

We hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: W. Ronald Robins, Registry No. 26,222; Steven R. Borgman, Registry No. 33,160; W. Scott Brown, Registry No. 32,968; Leslie J. Clark, Registry No. 34,800; Laura A. Crowe, Registry No. 35,850; G. Harvey Dunn, III, Registry No. 31,102; Alfred H. Evans, Registry No. 22,032; Kevin M. Hart, Registry No. 36,823; William L. LaFuze, Registry No. 27,205; Julian Clark Martin, Registry No. 26,198; Peter E. Mims, Registry No. 32,429; Jack R. Springgate, Registry No. 17,385; Darrell E. Warner, Registry No. 36,046; Karen Tucker White, Registry No. 34,267; of the firm of Vinson & Elkins L.L.P., 2700 First City Tower, 1001 Fannin

Street, Houston, Texas 77002-6760. Address all telephone calls to: W. Ronald Robins at (713) 758-2452. Address all correspondence to:

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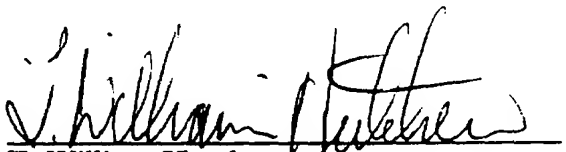
2918 First City Tower
1001 Fannin Street
Houston, Texas 77002-6067

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and may jeopardize the validity of the application or any patent issuing thereon.

By: 
T. William Hutchens
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Date: May 28, 1993

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I, Tai-Tung Yip, state that I am a citizen of Hong Kong. I was born in Mainland China in 1945 and thereafter immigrated to Hong Kong. The government of Hong Kong issued to me a Certificate of Identity. In 1988 I immigrated to the United States of America where I currently reside.

By: _____
Tai-Tung Yip
Inventor

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Post Office Address:
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Department of Pediatrics
Children's Nutrition Research Center
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Express Mail EE159324579US

# POWER OF ATTORNEY

BAYLOR COLLEGE OF MEDICINE, assignee of all right, title and interest in the enclosed patent application, hereby appoints the following agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Ronald G. Bliss, Reg. No. 28,691; Robert J. Koch, Reg. No. 26,637; Wayne E. Webb, Reg. No. 30,116; William A. Stout, Reg. No. 18,773; Paul L. Deverter, Reg. No. 19,747; James W. Repass, Reg. No. 30,487; Thomas D. Paul, Reg. No. 32,714; John M. Mings, Reg. No. 35,955; Mark E. Ungerman, Reg. No. 32,070; Peter J. Davis, Reg. No. 36,119; and Scott Denko, Reg. No. 37,606; Ross E. Davidson, Reg. No. P-41,698; David L. Fox, Reg. No. 40,612.

Address all correspondence to Patent Department; Fulbright & Jaworski L.L.P., 1301 McKinney, Suite 5100, Houston, Texas 77010-3095.

BAYLOR COLLEGE OF MEDICINE

W. Dalton Tomlin
Senior Vice President and General Counsel

DATE: July 24, 1998

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | |
|---|---|---|---|
| **Serial No.:** | **TBA** | **Docket No.:** | **D-5639-C4** |
| **Filing Date:** | **July 27, 1998** | | |
| **Applicants:** | **T. W. Hutchens et al.** | **Examiner:** | **TBA** |
| **Title:** | **Method and Apparatus for Desorption and Ionization of Analytes** | **Art Unit:** | **TBA** |

Assistant Commissioner for Patents
Washington, D.C. 20231

## PRELIMINARY AMENDMENTS

IN THE TITLE:

Please delete the title of the invention and insert therefor:

-- SURFACE-ENHANCED LASER DESORPTION/IONIZATION FOR DESORPTION AND DETECTION OF ANALYTES --

IN THE SPECIFICATION:

Please amend the specification as follows.

On Page 1, line 3, please insert:

-- This application is a continuation of co-pending application serial no. 08/068,896, filed May 28, 1993, the entire disclosure of which is incorporated by reference. --

On page 4, line 16, following "2299" insert -- -2301 --.

On page 4, line 19, change "(in press)" to -- 18:841-843 (1989) --.

On page 9, line 4, change "crosslineated" to -- cross-linked --.

On page 15, line 6, change "1A" to -- 1, top profile, --.

On page 15, line 7, change "1B" to -- 1, bottom profile --.

On page 15, line 9, change "2A" to -- 2, top profile --.

On page 15, line 10, change "2B" to -- 2, second from top profile and second from bottom profile, --.

On page 15, line 12, change "2D" to -- 2, bottom profile, --.

On page 15, line 19, change "5A" to -- 5, top profile, --.

On page 15, line 20, change "5B" to -- 5, middle profile, --.

On page 15, line 20, change "5C" to -- 5, bottom profile, --.

On page 16, line 3, change "7A" to -- 7, bottom profile, --.

On page 16, line 3, change "7B" to -- 7, second from bottom profile, --.

On page 16, line 5, change "7C" to -- 7, second from top profile, --.

On page 16, line 6, change "7D" to -- 7, top profile, --.

On page 16, line 8, change "(bottom)" to -- , bottom profile, --.

On page 16, line 9, change "(top)" to -- , top profile, --.

On page 16, line 11, change "paramagmetic" to -- paramagnetic --.

On page 16, line 19, change "12A" to -- 12, top profile, --.

On page 16, line 19, change "12B" to -- 12, bottom profile, --.

On page 17, line 1, change "14A" to -- 14, top profile, --.

On page 17, line 2, change "14B" to -- 14, bottom profile, --.

On page 17, line 5, change "16A" to -- 16, top profile, --.

On page 17, line 6, change "16B" to -- 16, bottom profile, --.

On page 17, line 9, change "linear" to -- linker --.

On page 17, line 10, following "18" please insert -- , top profile, --.

On page 17, line 11, change "18B" to -- 18, bottom profile, --.

On page 20, line 9, change "5A" to -- 5, top profile, --.

On page 20, line 12, change "5B" to -- 5, middle profile, --.

On page 20, line 14, following "adduct peaks." please insert -- FIGURE 5, bottom profile, --.

On page 26, line 2, change "21" to -- 18 --.

IN THE CLAIMS:

Please amend the claims as follows.

Cancel claims 2-31 without prejudice.

Add new claims 32-101 as follows:

-- 32. (New) A probe that is removably insertable into a mass spectrometer, the probe having a surface for presenting an analyte to an energy source that emits energy capable of desorbing the analyte from the probe for analyte detection, wherein at least the surface comprises a non-metallic material.

33. (New) The probe of claim 32 wherein the surface is adhered to the probe magnetically.

34. (New) The probe of claim 32 wherein the surface comprises metal, metal coated with a synthetic polymer, glass, ceramic, a synthetic polymer or a mixture thereof.

35. (New) The probe of claim 32 wherein the surface is coated with a synthetic polymer.

36. (New) The probe of claim 32 wherein the non-metallic material is substantially porous.

37. (New) The probe of claim 32 wherein the non-metallic material is substantially non-porous.

38. (New) The probe of claim 32 wherein the probe comprises stainless steel and the surface comprises a substantially porous material.

39. (New) The probe of claim 32 wherein the probe comprises stainless steel and the surface comprises a substantially non-porous material.

40. (New) The probe of claim 32 wherein the probe comprises glass.

41. (New) The probe of claim 32 wherein the probe comprises ceramic.

42. (New) The probe of claim 32 wherein the probe comprises a synthetic polymer.

43. (New) The probe of claim 36 wherein the porous material comprises sponge-like, polymeric, high surface areas.

44. (New) The probe of claim 37 wherein the non-porous material is selected from the group consisting of glass and polyacrylamide.

45. (New) The probe of claim 38 wherein the porous material comprises sponge-like, polymeric, high surface areas.

46. (New) The probe of claim 39 wherein the non-porous material is selected from the group consisting of glass and polyacrylamide.

47. (New) The probe of claim 43 wherein the porous material is selected from the group consisting of polypropylene, polystyrene, polyethylene, polycarbonate and nylon.

48. (New) The probe of claim 45 wherein the porous material is selected from the group consisting of polypropylene, polystyrene, polyethylene, polycarbonate and nylon.

49. (New) A method of desorbing an analyte from a probe surface comprising the steps of:

(a) providing a probe that is removably insertable into a mass spectrometer, the probe having a surface for presenting the analyte to an energy source that emits energy capable of desorbing the analyte from the probe for analyte detection, wherein at least the surface comprises a non-metallic material, and wherein the analyte is on the probe surface; and

(b) exposing the analyte to energy from the energy source, whereby the analyte is desorbed.

50. (New) The method of claim 49 wherein the energy source emits laser light that ionizes the analyte to produce an ion.

51. (New) The method of claim 49 further comprising after step (b) the steps of:

c) modifying the analyte chemically or enzymatically while deposited on the probe surface; and

d) repeating step (b).

52. (New) The method of claim 49 wherein the probe surface comprises an array of locations, each location having at least one analyte deposited thereon; and step (b) comprises desorbing a first analyte from a first location in the array;

and wherein the method further comprises the step of (c) desorbing a second analyte from a second location in the array.

53. (New) The method of claim 49 further comprising before step (b) the step of modifying the analyte chemically or enzymatically while deposited on the probe surface.

54. (New) The method of claim 49 wherein the surface comprises metal, metal coated with a synthetic polymer, glass, ceramic, a synthetic polymer or a mixture thereof.

55. (New) The method of claim 49 wherein the surface is coated with a synthetic polymer.

56. (New) The method of claim 49 wherein the non-metallic material is substantially porous.

57. (New) The method of claim 49 wherein the non-metallic material is substantially non-porous.

58. (New) The method of claim 49 wherein the probe comprises stainless steel and the surface comprises a substantially porous material.

59. (New) The method of claim 49 wherein the probe comprises stainless steel and the surface comprises a substantially non-porous material.

60. (New) The method of claim 49 wherein the probe comprises glass.

61. (New) The method of claim 49 wherein the probe comprises ceramic.

62. (New) The method of claim 49 wherein the probe comprises a synthetic polymer.

63. (New) The method of claim 49 wherein the analyte comprises protein.

64. (New) A system for detecting an analyte comprising:

a removably insertable probe having a surface for presenting the analyte to an energy source that emits energy capable of desorbing the analyte from the probe, wherein at least the surface comprises a non-metallic material, and an analyte on the surface;

an energy source that directs energy to the probe surface for desorbing the analyte; and

a detector in communication with the probe surface that detects the desorbed analyte.

65. (New) The system of claim 64 which is a laser desorption mass spectrometer wherein:

the energy source emits laser light that ionizes the analyte to produce an ion,

the system further comprises means for accelerating the ion to the detector,

the detector detects the ion, and

the system further comprises means for determining the mass of the ion.

66. (New) The system of claim 64 wherein the energy source emits laser light.

67. (New) The system of claim 64 wherein the energy source emits plasma energy or fast atoms.

68. (New) The system of claim 64 wherein the energy source emits energy of a variety of wavelengths.

69. (New) The system of claim 64 wherein the detector detects ions.

70. (New) The system of claim 64 wherein the detector detects radioactivity or light.

71. (New) The system of claim 64 further comprising means for accelerating the desorbed analyte to the detector.

72. (New) The system of claim 64 wherein the surface is adhered to the probe magnetically.

73. (New) The system of claim 64 wherein the surface comprises metal, metal coated with a synthetic polymer, glass, ceramic, a synthetic polymer or a mixture thereof.

74. (New) The system of claim 64 wherein the surface is coated with a synthetic polymer.

75. (New) The system of claim 64 wherein the non-metallic material is substantially porous.

76. (New) The system of claim 64 wherein the non-metallic material is substantially non-porous.

77. (New) The system of claim 64 wherein the probe comprises stainless steel and the surface comprises a substantially porous material.

78. (New) The system of claim 64 wherein the probe comprises stainless steel and the surface comprises a substantially non- porous material.

79. (New) The system of claim 64 wherein the probe comprises glass.

80. (New) The system of claim 64 wherein the probe comprises ceramic.

81. (New) The system of claim 64 wherein the probe comprises a synthetic polymer.

82. (New) The system of claim 75 wherein the porous material comprises sponge-like, polymeric, high surface areas.

83. (New) The system of claim 76 wherein the non-porous material is selected from the group consisting of glass and polyacrylamide.

84. (New) The system of claim 77 wherein the porous material comprises sponge-like, polymeric, high surface areas.

85. (New) The system of claim 78 wherein the non-porous material is selected from the group consisting of glass and polyacrylamide.

86. (New) A method for detecting an analyte comprising the steps of:

a) providing a system comprising:

(1) a removably insertable probe having a surface for presenting the analyte to an energy source that emits energy capable of desorbing the analyte from the probe, wherein at least the surface comprising a non-metallic material, and an analyte on the surface;

(2) an energy source that directs energy to the probe surface for desorbing the analyte; and

(3) a detector in communication with the probe surface that detects the desorbed analyte;

b) desorbing at least a portion of the analyte from the surface by exposing the analyte to the energy; and

c) detecting the desorbed analyte with the detector.

87. (New) The method of claim 86 wherein the system is a laser desorption mass spectrometer wherein the energy source emits laser light that ionizes the analyte to produce an ion, the detector detects the ion and the system further comprises means for accelerating the ion to the detector, and the method further comprises determining the mass of the ion.

88. (New) The method of claim 86 further comprising before step (b) the step of modifying the analyte chemically or enzymatically while deposited on the probe surface.

89. (New) The method of claim 86 further comprising after step (c) the steps of:

d) modifying the analyte chemically or enzymatically while deposited on the probe surface; and

e) repeating steps b) and c).

90. (New) The method of claim 86 wherein the probe surface comprises an array of locations, each location having at least one analyte deposited thereon; and step (b) comprises desorbing a first analyte from a first location in the array;

and wherein the method further comprises the step of:

d) desorbing a second analyte from a second location in the array; and

e) detecting the desorbed second analyte with the detector.

91. (New) The method of claim 87 further comprising the step of displaying the determined mass of the analyte.

92. (New) The method of claim 87 wherein the surface comprises metal, metal coated with a synthetic polymer, glass, ceramic, a synthetic polymer or a mixture thereof.

93. (New) The method of claim 87 wherein the surface is coated with a synthetic polymer.

94. (New) The method of claim 87 wherein the non-metallic material is substantially porous.

95. (New) The method of claim 87 wherein the non-metallic material is substantially non-porous.

96. (New) The method of claim 87 wherein the probe comprises stainless steel and the surface comprises a substantially porous material.

97. (New) The method of claim 87 wherein the probe comprises stainless steel and the surface comprises a substantially non-porous material.

98. (New) The method of claim 87 wherein the probe comprises glass.

99. (New) The method of claim 87 wherein the probe comprises ceramic.

100. (New) The method of claim 87 wherein the probe comprises a synthetic polymer.

101. (New) The method of claim 87 wherein the analyte comprises protein. --

IN THE ABSTRACT:

Please delete the abstract and replace it with the following:

-- This invention is directed to probes that are removably insertable into mass spectrometers. The probes have sample presenting surfaces, at least, that contain non-metallic materials. The probes are useful in methods of desorbing analytes from the probe surface. The invention also is directed to detection systems that include the probes and methods of detecting analytes using the system. --

## REMARKS

Claims 1-32 were originally filed. Applicants have canceled claims 2-31 and added new claims 32-101. Applicants make these amendments expressly without prejudice to their right to present claims corresponding to the cancelled or amended

claims in a timely filed continuing application. Therefore, claims 1 and 32-101 are pending and are presented for examination.

The claims add no new matter. Probes having surfaces, at least, comprising non-metallic materials finds support, e.g., on page 6, third paragraph; page 10, second full paragraph; and page 13, first full paragraph.

This application contains amino acid sequences. In a related application the Examiner stated that a sequence listing would not be required because the application did not claim any nucleotide or amino acid sequences. Applicants request the Examiner to confirm this in the next action. If the Examiner does not do so, Applicants will file a sequence listing and computer readable form after receiving the next office action.

## CONCLUSION

In view of the foregoing, Applicants request speedy examination of this application and the issuance of a formal Notice of Allowance.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone Thomas D. Paul at (713) 651-5325.

Applicants do not believe that any fees are due with this amendment. If, however, any additional fees are due, please charge these additional fees to the deposit account of Fulbright & Jaworski L.L.P., Account No. 06-23275 under Order No. 936611/D-5639-C4, from which the undersigned is authorized to draw.

Respectfully submitted,

Ross E. Davidson
Registration No. P-41,698
Counsel for Applicants

Date: 7/27/98
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